"Express Mail" mailing label number EF360328485US

Date of Deposit August 23, 2001

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR § 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231

Carolyn Roberts
(Typed or printed name of person mailing paper or fee)

Carolyn Roberts
(Signature of person mailing paper or fee)

PATENT
Case No. P-188

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
APPLICATION FOR UNITED STATES LETTERS PATENT

INVENTORS: Clark M. Whitehead
935 Lavera Road, Warminster, PA, 18974

Keith A. Earle
115 Embassy Drive, North Wales, PA 19454

Hector W. Alila
1268 Turnbury Lane, North Wales, PA 19454

W. Joseph Thompson
443 Maple Avenue, Doylestown, PA 18901

TITLE: METHODS FOR TREATMENT OF LUPUS ERYTHEMATOSUS

ATTORNEY: Robert W. Stevenson
Cell Pathways, Inc.
702 Electronic Drive
Horsham, Pennsylvania 19044
(215)-706-3800

METHODS FOR TREATMENT OF LUPUS ERYTHEMATOSUS

TECHNICAL FIELD

This invention relates to the treatment of lupus erythematosus.

BACKGROUND OF THE INVENTION

There are three types of lupus: drug-induced, discoid, and systemic. Drug-induced lupus occurs after the use of certain prescribed drugs. The symptoms of drug-induced lupus are similar to those of systemic lupus. The drugs most commonly connected with drug-induced lupus are hydralazine (used to treat high blood pressure or hypertension) and procainamide (used to treat irregular heart rhythms). Drug-induced lupus is more common in men who are given these drugs more often. However, not everyone who takes these drugs will develop drug-induced lupus. Only about 4 percent of the people who take these drugs will develop the antibodies suggestive of lupus. Of those 4 percent, only an extremely small number will develop overt drug-induced lupus. The symptoms usually fade when the medications are discontinued.

Discoid (cutaneous) lupus is limited to the skin. It is identified by a rash that may appear on the face, neck, and scalp. Discoid lupus is diagnosed by examining a biopsy of the rash. In discoid lupus the biopsy will show abnormalities that are not found in normal skin. Discoid lupus does not generally involve the body's internal organs, so it is a generally manageable disease.

In approximately 10 percent of patients, however, discoid lupus evolves into the more serious, systemic form of the disease, which can affect almost any organ or system of the body. Currently, this evolution cannot be predicted or prevented. Current treatment of discoid lupus will not prevent its progression to the systemic form. Individuals who progress to the systemic form are believed to have had systemic lupus at the outset, with the discoid rash as their main symptom.

For some people with systemic lupus, only the skin and joints will be involved. In others, the joints, lungs, kidneys, blood, or other organs and/or tissues may be affected. Generally, no two people with systemic lupus will have identical symptoms. Systemic lupus may include periods in which few, if any, symptoms are evident ("remission") and other times when the disease becomes more active ("flare").

The cause(s) of systemic lupus is unknown, but there are environmental and genetic factors involved. While scientists believe there is a genetic predisposition to the disease, it is known that environmental factors also play a role in triggering lupus. Some of the environmental factors that may trigger the disease are: infections, antibiotics (especially those in the sulfa and penicillin groups), ultraviolet light, extreme stress, certain drugs, and hormones.

Although lupus is known to occur within families, there is no known gene or genes that are thought to cause the illness.

For the vast majority of people with lupus, effective treatment can minimize symptoms, reduce inflammation, and maintain normal bodily functions. Preventive measures can reduce the risk of flares. For photosensitive patients, avoidance of (excessive) sun exposure and/or the regular application of sun screens can often prevent rashes. Regular exercise helps prevent muscle weakness and fatigue. Immunization protects against specific infections. Support groups, counseling, talking to family members, friends, and physicians can help alleviate the effects of stress. Needless to say, negative habits are hazardous to people with lupus. These include smoking, excessive consumption of alcohol, too much or too little of prescribed medication, or postponing regular medical checkups.

Treatment approaches are based on the specific needs and symptoms of each person. Because the characteristics and course of lupus may vary significantly among people, it is important to emphasize that a thorough medical evaluation and ongoing medical supervision are essential to ensure proper diagnosis and treatment.

There are no medications specifically approved by the F.D.A for the treatment of lupus. Nonetheless, medications are often prescribed "off label" by physicians for people with lupus, depending on which organ(s) are involved, and the severity of involvement. Commonly prescribed medications include non-steroidal anti-inflammatory drugs ("NSAIDs"), corticosteroids:

These medications are prescribed for a variety of rheumatic diseases, including lupus. Examples of such compounds include acetylsalicylic acid (e.g., aspirin), ibuprofen (Motrin), naproxen (Naprosyn), indomethacin (Indocin), nabumetone (Relafen), tolmetin (Tolectin), and a number of others. These drugs are usually recommended for muscle and joint pain, and arthritis. Aspirin and NSAIDs will cause gastric toxicities in some patients, particularly if used long-term. Patients

should be cautious about taking too much aspirin or NSAIDs since too many of these can reduce the blood flow to the kidney.

Corticosteroids (steroids) are hormones that have anti-inflammatory and immunoregulatory properties. They are normally produced in small quantities by the adrenal gland. This hormone controls a variety of metabolic functions in the body. Synthetically produced corticosteroids are used to reduce inflammation and suppress activity of the immune system. The most commonly prescribed drug of this type is Prednisone.

Because steroids have a variety of side effects, the dose has to be regulated to maximize the beneficial anti-immune/anti-inflammatory effects and minimize the negative side effects. Side effects occur more frequently when steroids are taken over long periods of time at high doses (for example, 60 milligrams of Prednisone taken daily for periods of more than one month). Such side effects include weight gain, a round face, acne, easy bruising, "thinning" of the bones (osteoporosis), high blood pressure, cataracts, onset of lupus erythematosus, increased risk of infection, stomach ulcers, hyperactivity, and an increase of appetite.

Antimalarials (e.g., chloroquine (Aralen) or hydroxychloroquine (Plaquenil)), commonly used in the treatment of malaria, may also be useful in some individuals with lupus. They are most often prescribed for skin and joint symptoms of lupus. It may take months before these drugs demonstrate a beneficial effect. Side effects are rare, and consist of occasional diarrhea or rashes. Some antimalarial drugs, such as quinine and chloroquine, can affect the eyes. Therefore, it is important to see an eye doctor (ophthalmologist) regularly. The manufacturer suggests an eye exam before starting the drug and one exam every six months thereafter. However, a physician might suggest a yearly exam is sufficient.

Immunomodulating drugs (e.g., azathioprine (Imuran) and cyclophosphamide (Cytoxan)) are in a group of agents known as cytotoxic or immunosuppressive drugs and have been prescribed to treat lupus. These drugs act in a similar manner to the corticosteroid drugs in that they suppress inflammation and tend to suppress the immune system. The side effects of these drugs include anemia, low white blood cell count, and increased risk of infection. Their use may also predispose an individual to developing cancer later in life.

Other agents like methotrexate and cyclosporin have been used to control the symptoms of lupus. Both are immunomodulating drugs which have their own side

effects. As a result, they drugs are still in the investigational phase for lupus. Some of these agents are used in conjunction with apheresis, a blood filtering treatment. Apheresis has been tried by itself in an effect to remove specific antibodies from the blood but the results have not been promising.

Newer agents are directed toward specific cells of the immune system. These include agents which block the production of specific antibodies like those against DNA, or agents which act to suppress the synthesis of antibodies through other mechanisms. Examples of this are intravenous immunoglobulin injections which are given on a regular basis to increase platelets (cells important to coagulation).

Thus, the treatment options for lupus patients are limited, particularly so in the case of drugs that can have some effect on altering the progression of lupus, as opposed to treating symptoms. One feature of the progression of lupus is the infiltration of macrophages into affected areas of the body. It is believed that reducing the presence of such macrophages will slow the progression of the disease because such macrophages are associated with damage to normal tissue in lupus patients.

SUMMARY OF THE INVENTION

This invention represents a novel therapy for treating patients (e.g., humans or companion animals) with lupus erythematosus without the substantial side effects of prior pharmaceutical approaches. Specifically, this invention involves the administration of an inhibitor of phosphodiesterase 2 ("PDE2") to a mammal in need of treatment for lupus erythematosus. Preferably, that inhibitor also inhibits phosphodiesterase 5 ("PDE5"). In narrower aspects of this invention, this invention involves the administration of compounds of Formula I below to a mammal in need of treatment for lupus erythematosus.

As explained below, compounds that inhibit PDE2 can cause activated macrophages to undergo apoptosis.

BRIEF DESCRIPTION OF THE FIGURES

The file of this patent contains at least one drawing executed in color. Copies of this patent with color drawing(s) will be provided by the Patent and Trademark Office upon request and payment of the necessary fee.

Figure 1 is a graph that compares the PDE2 and PDE5 mRNA levels in control and activated macrophages.

Figure 2 is a fluorescent microscope photomicrograph of control macrophages stained via indirect immunofluorescence to show basal level of PDE5 protein in the cells.

Figure 3 is a fluorescent microscope photomicrograph of activated macrophages stained via indirect immunofluorescence to show increased level of PDE5 protein in the cells.

Figure 4 is a fluorescent microscope photomicrograph of control macrophages stained via indirect immunofluorescence to show basal level of PDE2 protein in the cells.

Figure 5 is a fluorescent microscope photomicrograph of activated macrophages stained via indirect immunofluorescence to show increased level of PDE2 protein in the cells.

Figure 6 is a graph that illustrates cGMP and cAMP hydrolysis levels in activated and control macrophages.

Figure 7 is a graph that illustrates cGMP hydrolysis levels in protein lysates from activated and control macrophages.

Figure 8 is a digital image obtained with a fluorescent microscope of activated macrophages treated with a PDE2 inhibitor wherein the macrophages undergo apoptosis as reflected by the presence of active caspase 3 (red signal).

Figure 9 is a digital image obtained with a fluorescent microscope of control (vehicle only) macrophages revealing only low, background levels of apoptosis as reflected by the reduced presence of active caspase 3 (red signal).

Figure 10 is a digital image obtained with a fluorescent microscope of activated macrophages treated with a PDE4-specific inhibitor wherein the macrophages do not undergo substantial apoptosis as reflected by the substantial absence of active caspase 3 (red signal).

Figure 11 is a digital image obtained with a fluorescent microscope of activated macrophages treated with a PDE5-specific inhibitor wherein the

macrophages do not undergo substantial apoptosis as reflected by the substantial absence of active caspase 3 (red signal).

Figure 12 is a graph illustrating decreased TNFα levels in activated macrophages with exposure to a PDE2 inhibitor.

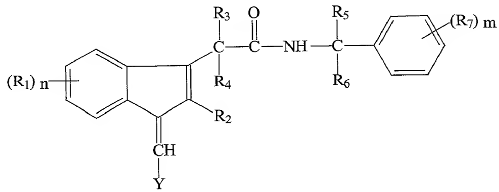
Figure 13 is a visual image of immunostaining revealing the expression of PDE2 protein in macrophages, lymphocytes and neutrophils in a patient with a known history of discoid lupus erythematosus (60x).

Figure 14 is a visual image of immunostaining revealing the expression of PDE2 protein in melanophages (macrophages) in the upper dermis of the skin in a patient with a known history of discoid lupus erythematosus (60x).

Figure 15 is a visual image of immunostaining revealing the expression of PDE5 protein in melanophages (macrophages) in the upper dermis of the skin in a patient with a known history of discoid lupus erythematosus (60x).

DETAILED DESCRIPTION OF THE INVENTION

As discussed above, the present invention includes the administration of an inhibitor of PDE2 to a mammal in need of treatment for lupus erythematosus. In addition, this invention includes the use of compounds of Formula I below (as well as their pharmaceutically acceptable salts) for treating a mammal with lupus erythematosus:



I

wherein R_1 is independently selected in each instance from the group consisting of hydrogen, halogen, lower alkyl, lower alkoxy, amino, lower alkylamino, di-lower alkylamino, lower alkylmercapto, lower alkyl sulfonyl, cyano, carboxamide , carboxylic acid, mercapto, sulfonic acid, xanthate and hydroxy;

R_2 is selected from the group consisting of hydrogen and lower alkyl;

R_3 is selected from the group consisting of hydrogen, halogen, amino, hydroxy, lower alkyl amino, and di-loweralkylamino;

R_4 is hydrogen, or R_3 and R_4 together are oxygen;

R_5 and R_6 are independently selected from the group consisting of hydrogen, lower alkyl, hydroxy-substituted lower alkyl, amino lower alkyl, lower alkylamino-lower alkyl, lower alkyl amino di-lower alkyl, lower alkyl nitrile, $-CO_2H$, $-C(O)NH_2$, and a C_2 to C_6 amino acid;

R_7 is independently selected in each instance from the group consisting of hydrogen, amino lower alkyl, lower alkoxy, lower alkyl, hydroxy, amino, lower alkyl amino, di-lower alkyl amino, halogen, $-CO_2H$, $-SO_3H$, $-SO_2NH_2$, and $-SO_2$(lower alkyl);

m and n are integers from 0 to 3 independently selected from one another;

Y is selected from the group consisting of quinolinyl, isoquinolinyl, pyridinyl, pyrimidinyl, pyrazinyl, imidazolyl, indolyl, benzimidazolyl, triazinyl, tetrazolyl,

thiophenyl, furanyl, thiazolyl, pyrazolyl, or pyrrolyl, or subsituted variants thereof wherein the substituents are one or two selected from the group consisting of halogen, lower alkyl, lower alkoxy, amino, lower alkylamino, di-lower alkylamino, hydroxy, -SO_2(lower alkyl) and -SO_2NH_2.

Preferred compounds of this invention for use with the methods described herein include those of Formula I where:

R_1 is selected from the group consisting of halogen, lower alkoxy, amino, hydroxy, lower alkylamino and di-loweralkylamino, preferably halogen, lower alkoxy, amino and hydroxy;

R_2 is lower alkyl;

R_3 is selected from the group consisting of hydrogen, halogen, hydroxy, amino, lower alkylamino and di-loweralkylamino, preferably, hydrogen, hydroxy and lower alkylamino;

R_5 and R_6 are independently selected from the group consisting of hydrogen, hydroxy-substituted lower alkyl, amino lower alkyl, lower alkylamino-lower alkyl, lower alkyl amino di-lower alkyl, -CO_2H, -C(O)NH_2; preferably hydrogen, hydroxy-substituted lower alkyl, lower alkyl amino di-lower alkyl, -CO_2H, and -C(O)NH_2;

R_7 is independently selected in each instance from the group consisting of hydrogen, lower alkoxy, hydroxy, amino, lower alkyl amino, di-lower alkyl amino, halogen, -CO_2H, -SO_3H, -SO_2NH_2, and -SO_2(lower alkyl); preferably hydrogen, lower alkoxy, hydroxy, amino, amino lower alkyl, halogen, -CO_2H, -SO_3H, -SO_2NH_2, and -SO_2(lower alkyl);

Preferably, at least one of the R_7 substituents is para- or ortho-located; most preferably ortho-located;

Y is selected from the group consisting of quinolinyl, isoquinolinyl, pyridinyl, pyrimidinyl and pyrazinyl or said substituted variants thereof.

Preferably, the substituents on Y are one or two selected from the group consisting of lower alkoxy, amino, lower alkylamino, di-lower alkylamino, hydroxy, -SO_2(lower alkyl) and -SO_2NH_2; most preferably lower alkoxy, di-lower alkylamino, hydroxy, -SO_2(lower alkyl) and -SO_2NH_2.

The present invention also is a method of treating a mammal with lupus by administering to a patient a pharmacologically effective amount of a pharmaceutical composition that includes a compound of Formula I, wherein R_1 through R_7 and Y are

as defined above. Preferably, this composition is administered without therapeutic amounts of an NSAID.

Compounds of this invention are inhibitors of phosphodiesterases PDE2. For convenience, the PDE inhibitory activity of such compounds can be tested as taught in U.S. patent application serial no. 09/046,739 filed March 24, 1998 to Pamukcu et al., which is incorporated herein by reference. Thus, compounds employed in this invention are useful inhibitors of PDE2 and preferably also PDE5. Most preferably, such compounds have an IC_{50} for PDE2 of no more than 25 μM.

Additional compounds besides those of Formula I can be identified for inhibitory effect on the activity of PDE2 and/or PDE5. Alternatively, cyclic nucleotide levels in whole cells are measured by radioimmunoassay ("RIA") and compared to untreated and drug-treated tissue samples and/or isolated enzymes.

Phosphodiesterase activity can be determined using methods known in the art, such as a method using radioactive ^{3}H cyclic GMP (cGMP)(cyclic 3',5'-guanosine monophosphate) as the substrate for the PDE enzyme. (Thompson, W.J., Teraski, W.L., Epstein, P.M., Strada, S.J., *Advances in Cyclic Nucleotide Research*, 10:69-92, 1979, which is incorporated herein by reference). In brief, a solution of defined substrate ^{3}H-cGMP specific activity (0.2 μM; 100,000 cpm; containing 40 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$ and 1 mg/mL BSA) is mixed with the drug to be tested in a total volume of 400μl. The mixture is incubated at 30°C for 10 minutes with isolated PDE2 and/or PDE5. Reactions are terminated, for example, by boiling the reaction mixture for 75 seconds. After cooling on ice, 100 μl of 0.5 mg/mL snake venom (*O. Hannah* venom available from Sigma) is added and incubated for 10 minutes at 30°C. This reaction is then terminated by the addition of an alcohol, e.g. 1 mL of 100% methanol. Assay samples are applied to 1 mL Dowex 1-X8 column; and washed with 1 mL of 100% methanol. The amount of radioactivity in the breakthrough and the wash from the column is combined and measured with a scintillation counter. The degree of phosphodiesterase inhibition is determined by calculating the amount of radioactivity in drug-treated reactions and comparing against a control sample (a reaction mixture lacking the tested compound but with drug solvent).

Alternatively, the ability of desirable compounds to inhibit the phosphodiesterases of this invention is reflected by an increase in cGMP in lupus erythematosus tissue

samples exposed to a compound being evaluated. The amount of PDE activity can be determined by assaying for the amount of cyclic GMP in the extract of treated cells using RIA. When PDE activity is evaluated in this fashion, a combined cGMP hydrolytic activity is assayed. The test compound is then incubated with the tissue at a concentration of compound between about 200 μM to about 200 pM. About 24 to 48 hours thereafter, the culture media is removed from the tissue, and the cells are solubilized. The reaction is stopped by using 0.2N HCl/50% MeOH. A sample is removed for protein assay. Cyclic GMP is purified from the acid/alcohol extracts of cells using anion-exchange chromatography, such as a Dowex column. The cGMP is dried, acetylated according to published procedures, such as using acetic anhydride in triethylamine, (Steiner, A.L., Parker, C.W., Kipnis, D.M., *J. Biol. Chem.,* 247(4):1106-13, 1971, which is incorporated herein by reference). The acetylated cGMP is quantitated using radioimmunoassay procedures (Harper, J., Brooker, G., *Advances in Nucleotide Research*, 10:1-33, 1979, which is incorporated herein by reference). Iodinated ligands (tyrosine methyl ester) of derivatized cyclic GMP are incubated with standards or unknowns in the presence of antisera and appropriate buffers. Antiserum may be produced using cyclic nucleotide-haptene directed techniques. The antiserum is from sheep injected with succinyl-cGMP-albumin conjugates and diluted 1/20,000. Dose-interpolation and error analysis from standard curves are applied as described previously (Seibert, A.F., Thompson, W.J., Taylor, A., Wilbourn, W.H., Barnard, J. and Haynes, J., *J. Applied Physiol.,* 72:389-395, 1992, which is incorporated herein by reference).

In addition, the tissue may be acidified, frozen (-70°C) and also analyzed for cGMP and cAMP.

More specifically as to tissue testing, the PDE inhibitory activity effect of a compound can also be determined from tissue biopsies obtained from humans or tissues from animals exposed to the test compound. A sample of tissue is homogenized in 500 μl of 6% trichloroacetic acid ("TCA"). A known amount of the homogenate is removed for protein analysis. The remaining homogenate is allowed to sit on ice for 20 minutes to allow for the protein to precipitate. Next, the homogenate is centrifuged for 30 minutes at 15,000g at 4°C. The supernatant is recovered, and the pellet recovered. The supernatant is washed four times with five volumes of water saturated diethyl ether. The upper ether layer is discarded between each wash. The aqueous ether extract is dried in a speed vac. Once dried, the sample can be frozen for future use, or used immediately. The dried

extract is dissolved in 500 μl of assay buffer. The amount of PDE inhibition is determined by assaying for the amount of cyclic nucleotides using RIA procedures as described above.

In addition to compounds disclosed herein, other compounds that inhibit both PDE2 and PDE5 include compounds disclosed in U.S. Patents Nos. 5,401,774 (e.g., exisulind), 6,063,818, 5,998,477, and 5,965,619. These patents are incorporated herein by reference.

When referring to an "a physiologically effective amount of an inhibitor of PDE2 and PDE5" we mean not only a single compound that inhibits those enzymes but a combination of several compounds, each of which can inhibit one or both of those enzymes. Single compounds that inhibit both enzymes are preferred.

When referring to an "inhibitor [that] does not substantially inhibit COX I or COX II," we mean that in the ordinary sense of the term. By way of example only, if the inhibitor has an IC_{50} for either PDE2 or PDE5 that is at least half of the IC_{50} of COXI and/or COXII, a drug achieving the PDE IC_{50} in the blood could be said not to substantially inhibit the COX enzymes. Preferably, the IC_{50} for the COX enzymes is in the order of 10 fold or more higher than the IC_{50} for PDE2/PDE5. Preferably, the IC_{50} for the COX enzymes is greater than about 40 μM.

As used herein, the term "halo" or "halogen" refers to chloro, bromo, fluoro and iodo groups, and the term "alkyl" refers to straight, branched or cyclic alkyl groups and to substituted aryl alkyl groups. The term "lower alkyl" refers to C_1 to C_8 alkyl groups.

The term "hydroxy-substituted lower alkyl" refers to lower alkyl groups that are substituted with at least one hydroxy group, preferably no more than three hydroxy groups.

The term "$-SO_2$(lower alkyl)" refers to a sulfonyl group that is substituted with a lower alkyl group.

The term "lower alkoxy" refers to alkoxy groups having from 1 to 8 carbons, including straight, branched or cyclic arrangements.

The term "lower alkylmercapto" refers to a sulfide group that is substituted with a lower alkyl group; and the term "lower alkyl sulfonyl" refers to a sulfone group that is substituted with a lower alkyl group.

The term "pharmaceutically acceptable salt" refers to non-toxic acid addition salts and alkaline earth metal salts of the compounds of Formula I. The salts can be prepared in situ during the final isolation and purification of such compounds, or separately by reacting the free base or acid functions with a suitable organic acid or base, for example. Representative acid addition salts include the hydrochloride, hydrobromide, sulfate, bisulfate, acetate, valerate, oleate, palmatate, stearate, laurate, borate, benzoate, lactate, phosphate, tosylate, mesylate, citrate, maleate, fumarate, succinate, tartrate, glucoheptonate, lactobionate, lauryl sulfate salts and the like. Representative alkali and alkaline earth metal salts include the sodium, calcium, potassium and magnesium salts.

It will be appreciated that certain compounds of Formula I can possess an asymmetric carbon atom and are thus capable of existing as enantiomers. Unless otherwise specified, this invention includes such enantiomers, including any racemates. The separate enaniomers may be synthesized from chiral starting materials, or the racemates can be resolved by conventional procedures that are well known in the art of chemistry such as chiral chromatography, fractional cyrstallization of diastereomeric salts and the like.

Compounds of Formula I also can exist as geometrical isomers (Z and E); the Z isomer is preferred.

Compounds of this invention may be formulated into pharmaceutical compositions together with pharmaceutically acceptable carriers for oral administration in solid or liquid form, or for rectal or topical administration, although carriers for oral administration are most preferred.

Pharmaceutically acceptable carriers for oral administration include capsules, tablets, pills, powders, troches and granules. In such solid dosage forms, the carrier can comprise at least one inert diluent such as sucrose, lactose or starch. Such carriers can also comprise, as is normal practice, additional substances other than diluents, *e.g.*, lubricating agents such as magnesium stearate. In the case of capsules, tablets, troches and pills, the carriers may also comprise buffering agents. Carriers such as tablets, pills and granules can be prepared with enteric coatings on the surfaces of the tablets, pills or granules. Alternatively, the enterically coated compound can be pressed into a tablet, pill, or granule, and the tablet, pill or granules for administration to the patient. Preferred enteric coatings include those that dissolve or disintegrate at colonic pH such as shellac or Eudraget S.

Pharmaceutically acceptable carriers include liquid dosage forms for oral administration, *e.g.,* pharmaceutically acceptable emulsions, solutions, suspensions, syrups and elixirs containing inert diluents commonly used in the art, such as water. Besides such inert diluents, compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, and sweetening, flavoring and perfuming agents.

Pharmaceutically acceptable carriers for topical administration include DMSO, alcohol or propylene glycol and the like that can be employed with patches or other liquid-retaining material to hold the medicament in place on the skin so that the medicament will not dry out.

Pharmaceutically acceptable carriers for rectal administration are preferably suppositories that may contain, in addition to the compounds of this invention excipients such as cocoa butter or a suppository wax, or gel.

The pharmaceutically acceptable carrier and compounds of this invention are formulated into unit dosage forms for administration to a patient. The dosage levels of active ingredient (*i.e.,* compounds of this invention) in the unit dosage may be varied so as to obtain an amount of active ingredient effective to achieve lesion-eliminating activity in accordance with the desired method of administration (*i.e.*, oral or rectal). The selected dosage level therefore depends upon the nature of the active compound administered, the route of administration, the desired duration of treatment, and other factors. If desired, the unit dosage may be such that the daily requirement for active compound is in one dose, or divided among multiple doses for administration, *e.g.*, two to four times per day.

The compounds of this invention can be formulated with pharmaceutically acceptable carriers into unit dosage forms in a conventional manner so that the patient in need of therapy for lupus erythematosus can periodically (*e.g.,* once or more per day) take a compound according to the methods of this invention. The exact initial dose of the compounds of this invention can be determined with reasonable experimentation. The initial dosage calculation would also take into consideration several factors, such as the formulation and mode of administration, e.g. oral or intravenous, of the particular compound. A total daily oral dosage of about 50 mg – 2.0 gr of such compounds would achieve a desired systemic circulatory concentration. As discussed below, an oral dose of about 800 mg/day has been found appropriate in mammals.

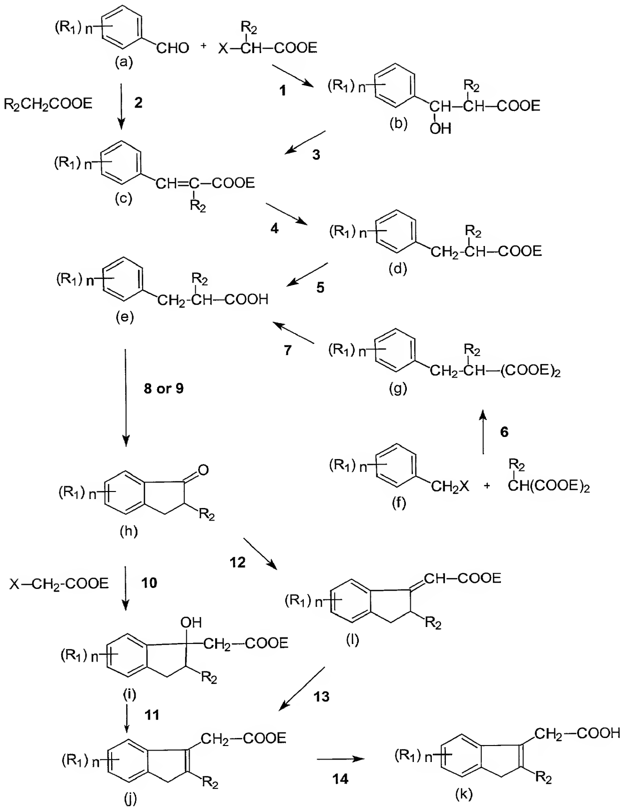
As explained below, PDE2/5 inhibitors within this invention cause apoptosis in the infiltrating, activated macrophages that are associated with damage to pancreatic β-islet cells. Because the diabetic patient continually produces activated macrophages, PDE2/5 inhibitors of this invention should be administered chronically, i.e., for at least two weeks at a time. In this manner, as any activated macrophages arise from monocytes, the drug in the patient's system can cause the macrophages to apoptose. In our hands, PDE2/5 inhibitors do cause monocytes to apoptose, given that animals exposed to such inhibitors chronically have normal monocyte blood counts.

The pharmaceutical compositions of this invention are preferably packaged in a container (*e.g.,* a box or bottle, or both) with suitable printed material (*e.g.,* a package insert) containing indications and directions for use in the treatment of lupus erythematosus, etc.

There are several general schemes for producing compounds of Formula I useful in this invention. One general scheme (which has several sub-variations) involves the case where both R_3 and R_4 are both hydrogen. This first scheme is described immediately below in Scheme I. The other general scheme (which also has several sub-variations) involves the case where at least one of R_3 and R_4 is a moiety other than hydrogen but within the scope of Formula I above. This second scheme is described below as "Scheme II."

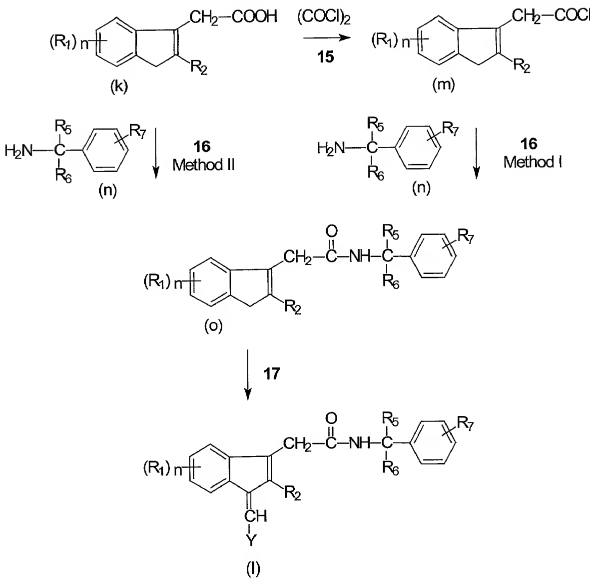
The general scheme for preparing compounds where both R_3 and R_4 are both hydrogen is illustrated in Scheme I, which is described in part in U.S. Patent No. 3,312,730, which is incorporated herein by reference. In Scheme I, R_1 is as defined in Formula I above. However, in Scheme I, that substituent can also be a reactive moiety (e.g. a nitro group) that later can be reacted to make a large number of other substituted indenes from the nitro-substituted indenes.

Scheme I



(R1) n
CHO
+
R2
X—CH—COOE
(a)
1
(R1) n
R2
CH—CH—COOE
(b)
OH
R2CH2COOE
2
3
(R1) n
CH=C—COOE
(c)
R2
4
(R1) n
R2
CH2-CH—COOE
(d)
(R1) n
R2
CH2-CH—COOH
(e)
5
7
(R1) n
R2
CH2-CH—(COOE)2
(g)
8 or 9
6
(R1) n
CH2X
+
R2
CH(COOE)2
(f)
(R1) n
O
R2
(h)
12
X—CH2-COOE
10
(R1) n
CH—COOE
R2
(l)
OH
CH2—COOE
(R1) n
R2
(i)
11
13
(R1) n
CH2—COOE
R2
(j)
14
(R1) n
CH2—COOH
R2
(k)

Scheme I (continued)



In Scheme I, several sub-variations can be used. In one sub-variation, a substituted benzaldehyde (a) may be condensed with a substituted acetic ester in a Knoevenagel reaction (see reaction 2) or with an α-halogeno propionic ester in a Reformatsky Reaction (see reactions 1 and 3). The resulting unsaturated ester (c) is hydrogenated and hydrolyzed to give a substituted benzyl propionic acid (e) (see reactions 4 and 5). Alternatively, a substituted malonic ester in a typical malonic ester synthesis (see reactions 6 and 7) and hydrolysis decarboxylation of the resulting substituted ester (g) yields the benzyl propionic acid (e) directly. This latter method is especially preferable for nitro and alkylthio substituents on the benzene ring.

The next step is the ring closure of the β-aryl proponic acid (e) to form an indanone (h) which may be carried out by a Friedel-Crafts Reaction using a Lewis acid catalyst (Cf. Organic Reactions, Vol. 2, p. 130) or by heating with polyphosphoric acid (see reactions 8 and 9, respectively). The indanone (h) may be condensed with an α-halo ester in the Reformatsky Reaction to introduce the aliphatic acid side chain by replacing the carboxyl group (see reaction 10). Alternately, this introduction can be carried out by the use of a Wittig Reaction in which the reagent is a α-triphenylphosphinyl ester, a reagent that replaces the carbonyl with a double bond to the carbon (see reaction 12). This product (l) is then immediately rearranged into the indene (j)(see reaction 13). If the Reformatsky Reaction route is used, the intermediate 3-hydroxy-3-aliphatic acid derivative i must be dehydrated to the indene (j) (see reaction 11).

The indenylacetic acid (k) in THF then is allowed to react with oxalyl or thionyl chloride or similar reagent to produce the acid chloride (m) (see reaction 15), whereupon the solvent is evaporated. There are two methods to carry out reaction 16, which is the addition of the benzylamine side chain (n).

Method (I)

In the first method, the benzylamine (n) is added slowly at room temperature to a solution of 5-fluoro-2-methyl-3-indenylacetyl chloride in CH_2Cl_2. The reaction mixture is refluxed overnight, and extracted with aqueous HCl (10%), water, and aqueous $NaHCO_3$ (5%). The organic phase is dried (Na_2SO_4) and is evaporated to give the amide compound (o).

Method (II)

In the second method, the indenylacetic acid (k) in DMA is allowed to react with a carbodiimide (e.g. N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride) and benzylamine at room temperature for two days. The reaction mixture is added dropwise to stirred ice water. A yellow precipitate is filtered off, is washed with water, and is dried *in vacuo*. Recrystallization gives the amide compound (o).

Compounds of the type a' (Scheme III), o (Scheme I), t (Scheme II), y (Scheme IIB) may all be used in the condensation reaction shown in Scheme III.

Substituents

X = halogen, usually Cl or Br.

E = methyl, ethyl or benzyl, or lower acyl.

R_1, R_2, R_6, R_5, and R_7= as defined in Formula I.

Y, n and m = as defined in Formula I.

Reagents and general conditions for Scheme I (numbers refer to the numbered reactions):

(1) Zn dust in anhydrous inert solvent such as benzene and ether.

(2) $KHSO_4$ or p-toluene sulfonic acid.

(3) $NaOC_2H_5$ in anhydrous ethanol at room temperature.

(4) H_2 palladium on charcoal, 40 p.s.i. room temperature.

(5) NaOH in aqueous alcohol at 20 - 100°.

(6) $NaOC_2H_5$ or any other strong base such as NaH or K-t-butoxide.

(7) Acid.

(8) Friedel-Crafts Reaction using a Lewis Acid catalyst Cf. Organic Reactions, Vol. II, p. 130.

(9) Heat with polyphosphoric acid.

(10) Reformatsky Reaction: Zn in inert solvent, heat.

(11) p-Toluene sulfonic acid and $CaCl_2$ or I_2 at 200°

(12) Wittig Reaction using $(C_6H_5)_3$ P=C-COOE 20-80° in ether or benzene

(13) (a) NBS/CCl_4/benzoyl peroxide

(b) PtO_2/ H_2 (1 atm.)/acetic acid

(14) (a) NaOH

(b) HCl

(15) Oxalyl or thionyl chloride in CH_2Cl_2 or THF

(16) Method I: 2 equivalents of NH_2-$C(R_5R_6)$-Ph-$(R_7)_m$

Method II: carbodiimide in THF

(17) 1N $NaOCH_3$ in MeOH under reflux conditions

Indanones within the scope of compound (h) in Scheme I are known in the literature and are thus readily available as intermediates for the remainder of the synthesis so that reactions 1-7 can be conveniently avoided. Among such known indanones are:

5-methoxyindanone

6-methoxyindanone

5-methylindanone

5-methyl-6-methoxyindanone

5-methyl-7-chloroindanone

4-methoxy-7-chloroindanone

4-isopropyl-2,7-dimethylindanone

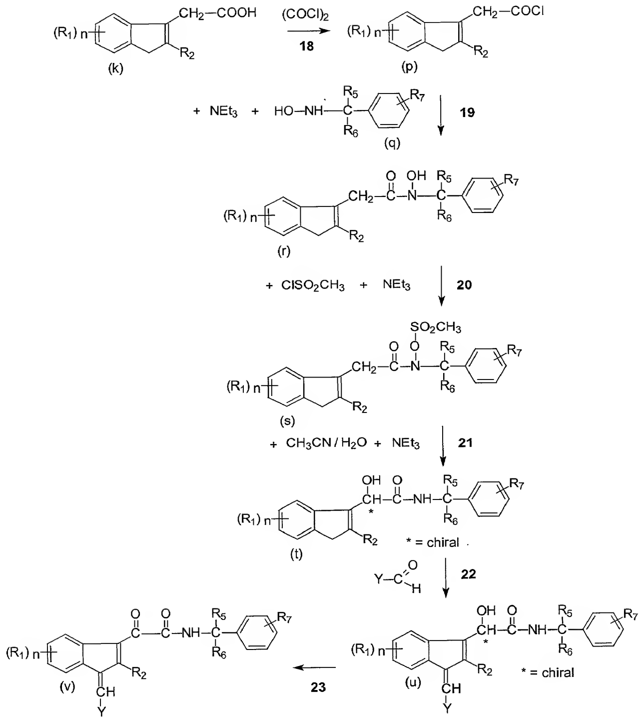
5,6,7-trichloroindanone

2-n-butylindanone

5-methylthioindanone

Scheme II has two mutually exclusive sub-schemes: Scheme IIA and Scheme II B. Scheme II A is used when R_3 is hydroxy and R_4 is hydrogen or when the two substituents form an oxo group. When R_3 is lower alkyl amino, Scheme II B is employed.

Scheme IIA



Similar to Scheme I, in Scheme IIA the indenylacetic acid (k) in THF is allowed to react with oxalylchloride under reflux conditions to produce the acid chloride (p) (see reaction 18), whereupon the solvent is evaporated. In reaction 19, a 0°C mixture of a benzyl hydroxylamine hydrochloride (q) and Et_3N is treated with a cold solution of the acid chloride in CH_2Cl_2 over a period of 45-60 minutes. The

mixture is warmed to room temperature and stirred for one hour, and is treated with water. The resulting organic layer is washed with 1 N HCl and brine, is dried over magnesium sulfate and is evaporated. The crude product, a N-hydroxy-N-benzyl acetamide (r) is purified by crystallization or flash chromatography. This general procedure is taught by Hoffman et al., JOC 1992, 57, 5700-5707.

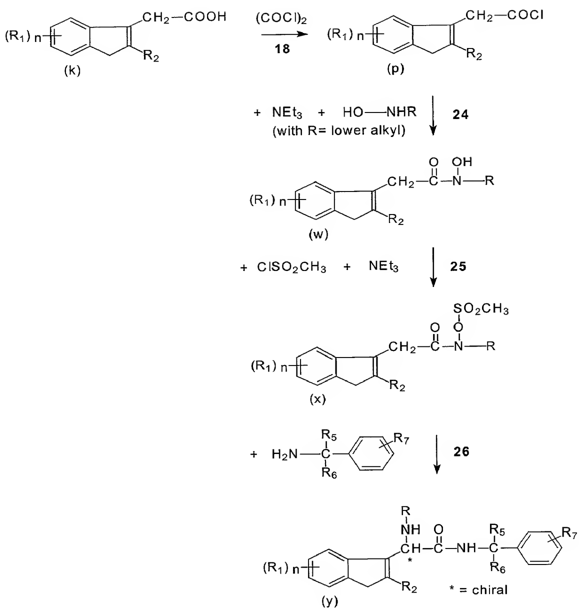
The next step is the preparation of the N-mesyloxy amide (s) in reaction 20, which is also taught by Hoffman et al., JOC 1992, 57, 5700-5707. Specifically, to a solution of the hydroxamic acid (r) in CH_2Cl_2 at 0°C is added triethylamine. The mixture is stirred for 10-12 minutes, and methanesulfonyl chloride is added dropwise. The mixture is stirred at 0°C for two hours, is allowed to warm to room temperature, and is stirred for another two hours. The organic layer is washed with water, 1 N HCl, and brine, and is dried over magnesium sulfate. After rotary evaporation, the product(s) is usually purified by crystallization or flash chromatography.

The preparation of the N-benzyl-α-(hydroxy) amide (t) in reaction 21, is also taught by Hoffman et al., JOC 1992, 57, 5700-5707 and Hoffman et al., JOC 1995, 60, 4121-4125. Specifically, to a solution of the N-(mesyloxy) amide (s) in CH_3CN/H_2O is added triethylamine in CH_3CN over a period of 6-12 hours. The mixture is stirred overnight. The solvent is removed, and the residue is dissolved in ethyl acetate. The solution is washed with water, 1 N HCl, and brine, and is dried over magnesium sulfate. After rotary evaporation, the product (t) is usually purified by recrystallization.

Reaction 22 in Scheme IIA involves a condensation with certain aldehydes, which is described in Scheme III below, a scheme that is common to products made in accordance with Schemes I, IIA and IIB.

The final reaction 23 in Scheme IIA is the preparation of the N-benzyl-α-ketoamide (v), which involves the oxidation of a secondary alcohol (u) to a ketone by e.g., a Pfitzner-Moffatt oxidation, which selectively oxidizes the alcohol without oxidizing the Y group. Compounds (u) and (v) may be derivatized to obtain compounds with R_3 and R_4 groups as set forth in Formula I.

Scheme IIB



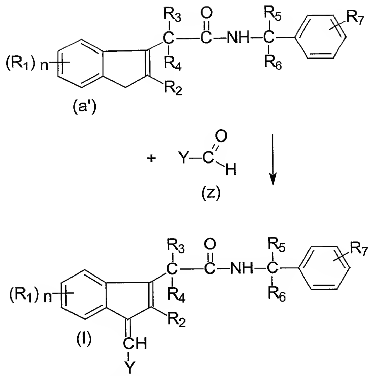
As explained above, Scheme IIB is employed when R_3 is lower alkyl amino. Similar to Scheme I, in Scheme IIB the indenylacetic acid (k) in THF is allowed to react with oxalylchloride under reflux conditions to produce the acid chloride (p) (see reaction 18), whereupon the solvent is evaporated. In reaction 24, a mixture of an alkyl hydroxylamine hydrochloride (i.e. HO-NHR where R is a lower alkyl, preferably isopropyl) and Et_3N is treated at 0° C with a cold solution of the acid

chloride in CH_2Cl_2 over a period of 45-60 minutes. The mixture is warmed to room temperature and is stirred for one hour, and is diluted with water. The resulting organic layer is washed with 1 N HCl and brine, is dried over magnesium sulfate and is evaporated. The crude product, a N-hydroxy-N-alkyl acetamide (w) is purified by crystallization or flash chromatography. This general procedure is also taught by Hoffman et al., JOC 1992, 57, 5700-5707

The preparation of the N-mesyloxy amide (x) in reaction 25, which is also taught by Hoffman et al., JOC 1992, 57, 5700-5707. Specifically, a solution of the hydroxamic acid (w) in CH_2Cl_2 at 0°C is treated with triethylamine, is stirred for 10-12 minutes, and is treated dropwise with methanesulfonyl chloride. The mixture is stirred at 0°C for two hours, is allowed to warm to room temperature, and is stirred for another two hours. The resulting organic layer is washed with water, 1 N HCl, and brine, and is dried over magnesium sulfate. After rotary evaporation, the product (x) is usually purified by crystallization or flash chromatography.

The preparation of the N-benzyl indenyl-α-loweralkylamino- acetamide compound (y) in Scheme IIB as taught by Hoffman et al., JOC 1995, 60, 4121-25 and J. Am. Chem Soc. 1993, 115, 5031-34, involves the reaction of the N-mesyloxy amide (x), with a benzylamine in CH_2Cl_2 at 0°C is added over a period of 30 minutes. The resulting solution is stirred at 0°C for one hour and at room temperature overnight. The solvent is removed, and the residue is treated with 1 N NaOH. The extract with CH_2Cl_2 is washed with water and is dried over magnesium sulfate. After rotary evaporation, the product (y) is purified by flash chromatography or crystallization.

Scheme III



Scheme III involves the condensation of the heterocycloaldehydes (i.e., Y-CHO) with the indenyl amides to produce the final compounds of Formula I. This condensation is employed, for example, in reaction 17 in Scheme I above and in reaction 22 in Scheme IIA. It is also used to convert compound (y) in Scheme IIB to final compounds of Formula I.

In Scheme III, the amide (a') from the above schemes, an N-heterocycloaldehyde (z), and sodium methoxide (1 M in methanol) are stirred at 60°C under nitrogen for 24 hours. After cooling, the reaction mixture is poured into ice water. A solid is filtered off, is washed with water, and is dried in vacuo. Recrystallization provides a compound of Formula I in Schemes I and IIB and the intermediate (u) in Scheme IIA.

As has been pointed out above, it is preferable in the preparation of many types of the compounds of this invention, to use a nitro substituent on the benzene ring of the indanone nucleus and convert it later to a desired substituent since by this route a great many substituents can be reached. This is done by reduction of the nitro to the amino group followed by use of the Sandmeyer reaction to introduce chlorine, bromine, cyano or xanthate in place of the amino. From the cyano derivatives, hydrolysis yields the carboxamide and carboxylic acid; other derivatives of the carboxy group such as the esters can then be prepared. The xanthates, by hydrolysis,

yield the mercapto group that may be oxidized readily to the sulfonic acid or alkylated to an alkylthio group that can then be oxidized to alkylsulfonyl groups. These reactions may be carried out either before or after the introduction of the 1-substituent.

The foregoing may be better understood from the following examples that are presented for purposes of illustration and are not intended to limit the scope of the invention. As used in the following examples, the references to substituents such as R_1, R_2, etc., refer to the corresponding compounds and substituents in Formula I above.

EXAMPLE 1

(Z)-5-Fluoro-2-Methyl-(4-Pyridinylidene)-3-(N-Benzyl)-Indenylacetamide

(A) p-Fluoro-α-methylcinnamic acid

p-Fluorobenzaldehyde (200 g, 1.61 mol), propionic anhydride (3.5 g, 2.42 mol) and sodium propionate (155 g, 1.61 mol) are mixed in a one liter three-necked flask which had been flushed with nitrogen. The flask is heated gradually in an oil-bath to 140°C. After 20 hours, the flask is cooled to 100°C and poured into 8 l of water. The precipitate is dissolved by adding potassium hydroxide (302 g) in 2 l of water. The aqueous solution is extracted with ether, and the ether extracts are washed with potassium hydroxide solution. The combined aqueous layers are filtered, are acidified with concentrated HCl, and are filtered. The collected solid, p-fluoro-α-methylcinnamic acid, is washed with water, and is dried and used as obtained.

(B) p-Fluoro-α-methylhydrocinnamic acid

To p-fluoro-α-methylcinnamic acid (177.9 g, 0.987 mol) in 3.6 l ethanol is added 11.0 g of 5% Pd/C. The mixture is reduced at room temperature under a hydrogen pressure of 40 p.s.i. When hydrogen uptake ceases, the catalyst is filtered off, and the solvent is evaporated in vacuo to give the product, p-fluoro-α-methylhydrocinnamic acid, which was used directly in the next step.

(C) 6-Fluoro-2-methylindanone

To 932 g polyphosphoric acid at 70°C (steam bath) is added p-fluoro-α-methylhydrocinnamic acid (93.2 g, 0.5 mol) slowly with stirring. The temperature is gradually raised to 95°C, and the mixture is kept at this temperature for 1 hour. The mixture is allowed to cool and is added to 2 l. of water. The aqueous suspension is extracted with ether. The extract is washed twice with saturated sodium chloride

solution, 5% Na_2CO_3 solution, and water, and is dried, and is concentrated on 200 g silica-gel; the slurry is added to a five pound silica-gel column packed with 5% ether-petroleum ether. The column is eluted with 5-10% ether-petroleum ether, to give 6-fluoro-2-methylindanone. Elution is followed by TLC.

(D) 5-fluoro-2-methylindenyl-3-acetic acid

A mixture of 6-fluoro-2-methylindanone (18.4 g, 0.112 mol), cyanoacetic acid (10.5 g, 0.123 mol), acetic acid (6.6 g), and ammonium acetate (1.7 g) in dry toluene (15.5 ml) is refluxed with stirring for 21 hours, as the liberated water is collected in a Dean Stark trap. The toluene is evaporated, and the residue is dissolved in 60 ml of hot ethanol and 14 ml of 2.2 N aqueous potassium hydroxide solution. 22 g of 85% KOH in 150 ml of water is added, and the mixture refluxed for 13 hours under nitrogen. The ethanol is removed under vacuum, and 500 ml water is added. The aqueous solution is extracted well with ether, and is then boiled with charcoal. The aqueous filtrate is acidified to pH 2 with 50% cold hydrochloric acid. The precipitate is dried and 5-fluoro-2-methylindenyl-3-acetic acid (M.P. 164-166°C) is obtained.

(E) 5-fluoro-2-methylindenyl-3-acetyl chloride

5-fluoro-2-methylindenyl-3-acetic acid (70 mmol) in THF (70 ml) is allowed to react with oxalylchloride (2 M in CH_2Cl_2; 35 ml; 70 mmol) under reflux conditions (24 hours). The solvent is evaporated to yield the title compound, which is used as such in the next step.

(F) 5-Fluoro-2-methyl-3-(N-benzyl)-indenylacetamide

Benzylamine (5 mmol) is added slowly at room temperature to a solution of 5-fluoro-2-methylindenyl-3-acetyl chloride (2.5 mmol.) in CH_2Cl_2 (10 ml). The reaction mixture is refluxed overnight, and is extracted with aqueous HCl (10%), water, and aqueous $NaHCO_3$ (5%). The organic phase is dried (Na_2SO_4) and is evaporated to give the title compound, which is recrystallized from CH_2Cl_2 to give the title compound as a white solid (m.p. 144°C).

(G) (Z)-5-Fluoro-2-methyl-(4-pyridinylidene)-3-(N-benzyl)-indenylacetamide

5-fluoro-2-methyl-3-(N-benzyl)-indenylacetamide (3.38 mmol), 4-pyridinecarboxaldehyde (4 mmol), sodium methoxide (1M $NaOCH_3$ in methanol (30 ml)) are heated at 60° C under nitrogen with stirring for 24 hours. After cooling, the reaction mixture is poured into ice water (200 ml). A solid is filtered off, washed with water, and dried in vacuo. Recrystallization from CH_3CN gives the title compound

(m.p. 202° C) as a yellow solid (R_1 = F, R_2 = CH_3, R_3 = H, R_4 = H, R_5 = H, R_6 = H, R_7 = H, n = 1, m = 1, Y = 4-pyridinyl).

(H) (E)-5-Fluoro-2-methyl-(4-pyridinylidene)-3-(N-benzyl)-indenylacetamide

The mother liquor obtained from the CH_3CN recrystallization of 1G is rich on the geometrical isomer of 1G. The E-isomer can be obtained pure by repeated recrystallizations from CH_3CN.

EXAMPLE 2

(Z)-5-Fluoro-2-Methyl-(3-Pyridinylidene)-3-(N-Benzyl)-Indenylacetamide

This compound is obtained from 5-fluoro-2-methyl-3-(N-benzyl)-indenylacetamide (Example 1F) using the procedure of Example 1, part G and replacing 4-pyridinecarboxaldehyde with 3-pyridinecarboxaldehyde. Recrystallization from CH_3CN gives the title compound (m.p. 175° C)(R_1 = F, R_2 = CH_3, R_3 = H, R_4 = H, R_5 = H, R_6 = H, R_7 = H, n = 1, m = 1, Y = 3-pyridinyl).

EXAMPLE 3

(Z)-5-Fluoro-2-Methyl-(2-Pyridinylidene)-3-(N-Benzyl)-Indenylacetamide

This compound is obtained from 5-fluoro-2-methyl-3-(N-benzyl)-indenylacetamide (Example 1F) using the procedure of Example 1, part G and replacing 4-pyridinecarboxaldehyde with 2-pyridinecarboxaldehyde. Recrystallization from ethylacetate gives the title compound (m.p. 218° C)(R_1 = F, R_2 = CH_3, R_3 = H, R_4 = H, R_5 = H, R_6 = H, R_7 = H, n = 1, m = 1, Y = 2-pyridinyl).

EXAMPLE 4

(Z)-5-Fluoro-2-Methyl-(4-Quinolinylidene)-3-(N-Benzyl)-Indenylacetamide

This compound is obtained from 5-fluoro-2-methyl-3-(N-benzyl)-indenylacetamide (Example 1F) using the procedure of Example 1, part G and replacing 4-pyridinecarboxaldehyde with 4-quinolinecarboxaldehyde. Recrystallization from ethylacetate gives the title compound (m.p. 239° C)(R_1 = F, R_2 = CH_3, R_3 = H, R_4 = H, R_5 = H, R_6 = H, R_7 = H, n = 1, m = 1, Y = 4-quinolinyl).

EXAMPLE 5

(Z)-5-Fluoro-2-Methyl-(4,6-Dimethyl-2-Pyridinylidene)-3-(N-Benzyl)-Indenylacetamide

5-Fluoro-2-methyl-3-(N-benzyl)-indenylacetamide from Example 1, part F is allowed to react with 4,6-dimethyl-2-pyridinecarboxaldehyde according to the procedure of Example 1, part G in order to obtain the title compound.

Recrystallization gives the title compound (R_1 = F, R_2 =CH_3, R_3 = H, R_4 = H, R_5 = H, R_6 = H, R_7 = H, n = 1, m = 1, Y = 4,6-dimethyl-2-pyridinyl).

EXAMPLE 6

(Z)-5-Fluoro-2-Methyl-(3-Quinolinylidene)-3-(N-Benzyl)-Indenylacetamide

5-Fluoro-2-methyl-3-(N-benzyl)-indenylacetamide from Example 1, part F is allowed to react with 3-quinolinecarboxaldehyde according to the procedure of Example 1, part G in order to obtain the title compound. Recrystallization gives the title compound (R_1 = F, R_2 = CH_3, R_3 = H, R_4 = H, R_5 = H, R_6 = H, R_7 = H, n = 1, m =1, Y = 3-quinolinyl)

EXAMPLE 7

(Z)-5-Fluoro-2-Methyl-(2-Quinolinylidene)-3-(N-Benzyl)-Indenylacetamide

5-Fluoro-2-methyl-3-(N-benzyl)-indenylacetamide from Example 1, part F is allowed to react with 2-quinolinecarboxaldehyde according to the procedure of Example 1, part G in order to obtain the title compound. Recrystallization gives the title compound (R_1 = F, R_2 = CH_3, R_3 = H, R_4 = H, R_5 = H, R_6 = H, R_7 = H, n = 1, m =1, Y = 2-quinolinyl).

EXAMPLE 8

(Z)-5-Fluoro-2-Methyl-(Pyrazinylidene)-3-(N-Benzyl)-Indenylacetamide

5-Fluoro-2-methyl-3-(N-benzyl)-indenylacetamide from Example 1, part F is allowed to react with pyrazinealdehyde according to the procedure of Example 1, part G in order to obtain the title compound. Recrystallization gives the title compound (R_1 = F, R_2 = CH_3, R_3 = H, R_4 = H, R_5 = H, R_6 = H, R_7 = H, n = 1, m =1, Y = pyrazinyl).

EXAMPLE 9

(Z)-5-Fluoro-2-Methyl-(3-Pyridazinylidene)-3-(N-Benzyl)-Indenylacetamide

5-Fluoro-2-methyl-3-(N-benzyl)-indenylacetamide from Example 1, part F is allowed to react with pyridazine-3-aldehyde according to the procedure of Example 1, part G in order to obtain the title compound. Recrystallization gives the title compound (R_1 = F, R_2 = CH_3, R_3 = H, R_4 = H, R_5 = H, R_6 = H, R_7 = H, n = 1, m =1, Y = 3-pyridazinyl).

EXAMPLE 10

(Z)-5-Fluoro-2-Methyl-(4-Pyrimidinylidene)-3-(N-Benzyl)-Indenylacetamide

5-Fluoro-2-methyl-3-(N-benzyl)-indenylacetamide from Example 1, part F is allowed to react with pyrimidine-4-aldehyde according to the procedure of Example

1, part G in order to obtain the title compound. Recrystallization gives the title compound (R_1 = F, R_2 = CH_3, R_3 = H, R_4 = H, R_5 = H, R_6 = H, R_7 = H, n = 1, m =1, Y = 4-pyrimidinyl).

EXAMPLE 11

(Z)-5-Fluoro-2-Methyl-(2-Methyl-4-Pyrimidinylidene)-3-(N-Benzyl)-Indenylacetamide

5-Fluoro-2-methyl-3-(N-benzyl)-indenylacetamide from Example 1, part F is allowed to react with 2-methyl-pyrimidine-4-aldehyde according to the procedure of Example 1, part G in order to obtain the title compound. Recrystallization gives the title compound (R_1 = F, R_2 = CH_3, R_3 = H, R_4 = H, R_5 = H, R_6 = H, R_7 = H, n = 1, m =1, Y = 2-methyl-4-pyrimidinyl).

EXAMPLE 12

(Z)-5-Fluoro-2-Methyl-(4-Pyridazinylidene)-3-(N-Benzyl)-Indenylacetamide

5-Fluoro-2-methyl-3-(N-benzyl)-indenylacetamide from Example 1, part F is allowed to react with pyridazine-4-aldehyde according to the procedure of Example 1, part G in order to obtain the title compound. Recrystallization gives the title compound (R_1 = F, R_2 = CH_3, R_3 = H, R_4 = H, R_5 = H, R_6 = H, R_7 = H, n = 1, m =1, Y = 4-pyridazinyl).

EXAMPLE 13

(Z)-5-Fluoro-2-Methyl-(1-Methyl-3-Indolylidene)-3-(N-Benzyl)-Indenylacetamide

5-Fluoro-2-methyl-3-(N-benzyl)-indenylacetamide from Example 1, part F is allowed to react with 1-methylindole-3-carboxaldehyde according to the procedure of Example 1, part G in order to obtain the title compound. Recrystallization gives the title compound (R_1 = F, R_2 = CH_3, R_3 = H, R_4 = H, R_5 = H, R_6 = H, R_7 = H, n = 1, m =1, Y = 1-methyl-3-indolyl).

EXAMPLE 14

(Z)-5-Fluoro-2-Methyl-(1-Acetyl-3-Indolylidene)-3-(N-Benzyl)-Indenylacetamide

5-Fluoro-2-methyl-3-(N-benzyl)-indenylacetamide from Example 1, part F is allowed to react with 1-acetyl-3-indolecarboxaldehyde according to the procedure of Example 1, part G in order to obtain the title compound. Recrystallization gives the title compound (R_1 = F, R_2 = CH_3, R_3 = H, R_4 = H, R_5 = H, R_6 = H, R_7 = H, n = 1, m =1, Y = 1-acetyl-3-indolyl).

EXAMPLE 15

(Z)-5-Fluoro-2-Methyl-(4-Pyridinylidene)-3-(N-2-Fluorobenzyl)-Indenylacetamide

(A) 5-Fluoro-2-methyl-3-(N-2-fluorobenzyl)-indenylacetamide

This compound is obtained from 5-fluoro-2-methylindenyl-3-acetyl chloride (Example 1E) using the procedure of Example 1, Part F and replacing benzylamine with 2-fluorobenzylamine.

(B) (Z)-5-Fluoro-2-methyl-(4-pyridinylidene)-3-(N-2-fluorobenzyl)-indenylacetamide

5-Fluoro-2-methyl-3-(N-2-fluorobenzyl)-indenylacetamide is allowed to react with 4-pryidinecarboxaldehyde according to the procedure of Example 1, part G in order to obtain the title compound. Recrystallization gives the title compound (R_1 = F, R_2 = CH_3, R_3 = H, R_4 = H, R_5 = H, R_6 = H, R_7 = F, n = 1, m =1, Y = 4-pyridinyl).

EXAMPLE 16

(Z)-5-Fluoro-2-Methyl-(3-Pyridinylidene)-3-(N-2-Fluorobenzyl)-Indenylacetamide

5-Fluoro-2-methyl-3-(N-2-fluorobenzyl)-indenylacetamide from Example 15, part A is allowed to react with 3-pryidinecarboxaldehyde according to the procedure of Example 1, part G in order to obtain the title compound. Recrystallization gives the title compound (R_1 = F, R_2 = CH_3, R_3 = H, R_4 = H, R_5 = H, R_6 = H, R_7 = F, n = 1, m =1, Y = 3-pyridinyl).

EXAMPLE 17

(Z)-5-Fluoro-2-Methyl-(2-Pyridinylidene)-3-(N-2-Fluorobenzyl)-Indenylacetamide

5-Fluoro-2-methyl-3-(N-2-fluorobenzyl)-indenylacetamide from Example 15, part A is allowed to react with 2-pyridinecarboxaldehyde according to the procedure of Example 1, part G in order to obtain the title compound. Recrystallization gives the title compound (R_1 = F, R_2 = CH_3, R_3 = H, R_4 = H, R_5 = H, R_6 = H, R_7 = F, n = 1, m =1, Y = 2-pyridinyl).

EXAMPLE 18

(Z)-5-Fluoro-2-Methyl-(4-Quinolinylidene)-3-(N-2-Fluorobenzyl)-Indenylacetamide

5-Fluoro-2-methyl-3-(N-2-fluorobenzyl)-indenylacetamide from Example 15, part A is allowed to react with 4-quinolinecarboxaldehyde according to the procedure of Example 1, part G in order to obtain the title compound. Recrystallization gives the title compound (R_1 = F, R_2 = CH_3, R_3 = H, R_4 = H, R_5 = H, R_6 = H, R_7 = F, n = 1, m =1, Y = 3-quinolinyl).

EXAMPLE 19

(Z)-5-Fluoro-2-Methyl-(3-Pyrazinylidene)-3-(N-2-Fluorobenzyl)-Indenylacetamide

5-Fluoro-2-methyl-3-(N-2-fluorobenzyl)-indenylacetamide from Example 15, part A is allowed to react with pyrazinealdehyde according to the procedure of Example 1, Part G in order to obtain the title compound. Recrystallization gives the title compound (R_1 = F, R_2 = CH_3, R_3 = H, R_4 = H, R_5 = H, R_6 = H, R_7 = F, n = 1, m =1, Y = 3-pyrazinyl).

EXAMPLE 20

(Z)-5-Fluoro-2-Methyl-(3-Pyridazinylidene)-3-(N-2-Fluorobenzyl)-Indenylacetamide

5-Fluoro-2-methyl-3-(N-2-fluorobenzyl)-indenylacetamide from Example 15, part A is allowed to react with 3-pryidaziine-3-aldehyde according to the procedure of Example 1, Part G in order to obtain the title compound. Recrystallization gives the title compound (R_1 = F, R_2 = CH_3, R_3 = H, R_4 = H, R_5 = H, R_6 = H, R_7 = F, n = 1, m =1, Y = 3-pyridazinyl).

EXAMPLE 21

(Z)-5-Fluoro-2-Methyl-(3-Pyrimidinylidene)-3-(N-2-Fluorobenzyl)-Indenylacetamide

5-Fluoro-2-methyl-3-(N-2-fluorobenzyl)-indenylacetamide from Example 15, part A is allowed to react with pryimidine-4-aldehyde according to the procedure of Example 1, Part G in order to obtain the title compound. Recrystallization gives the title compound (R_1 = F, R_2 = CH_3, R_3 = H, R_4 = H, R_5 = H, R_6 = H, R_7 = F, n = 1, m =1, Y = 3-pyrimidinyl).

EXAMPLE 22

(Z)-5-Fluoro-2-Methyl-(4-Pyridazinylidene)-3-(N-2-Fluorobenzyl)-Indenylacetamide

5-Fluoro-2-methyl-3-(N-2-fluorobenzyl)-indenylacetamide from Example 15, part A is allowed to react with pryidazine-4-aldehyde according to the procedure of Example 1, Part G in order to obtain the title compound. Recrystallization gives the title compound (R_1 = F, R_2 = CH_3, R_3 = H, R_4 = H, R_5 = H, R_6 = H, R_7 = F, n = 1, m =1, Y = 4-pyridazinyl).

EXAMPLE 23

(Z)-5-Fluoro-2-Methyl-(4-Pyridinylidene)-3-(N-(S-α-Hydroxymethyl)Benzyl)-Indenylacetamide

(A) 5-Fluoro-2-methyl-3-(N-(S-α-hydroxylmethyl)benzyl)-indenylacetamide

5-Fluoro-2-methylindenyl-3-acetic acid (from Example 1D) (2.6 mmol) in DMA (2 ml) is allowed to react with n-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (4 mmol) and S-2-amino-2-phenylethanol (3.5 mmol) at room temperature for two days. The reaction mixture is added dropwise to stirred ice water (50 ml). A white precipitate is filtered off, washed with water (5 ml), and dried in vacuo. Recrystallization from ethylacetate gives the desired compound.

(B) (Z)-5-fluoro-2-methyl-(4-pyridinylidene)-3-(N-(S-α-hydroxymethyl)benzyl)-indenylacetamide

5-Fluoro-2-methyl-3-(N-(S-α-hydroxylmethyl)benzyl)-indenylacetamide from part A is allowed to react with 4-pryidinecarboxaldehyde according to the procedure of Example 1, Part G in order to obtain the title compound. Recrystallization gives the title compound (R_1 = F, R_2 = CH_3, R_3 = H, R_4 = H, R_5 = CH_2OH, R_6 = H, R_7 = H, n = 1, m =1, Y = 4-pyridinyl).

EXAMPLE 24

(Z)-5-Fluoro-2-Methyl-(3-Pyridinylidene)-3-(N-(S-α-Hydroxymethyl)Benzyl)-Indenylacetamide

5-Fluoro-2-methyl-3-(N-(S-α-hydroxylmethyl)benzyl)-indenylacetamide from Example 23 part A is allowed to react with 3-pryidinecarboxaldehyde according to the procedure of Example 1, Part G in order to obtain the title compound. Recrystallization gives the title compound (R_1 = F, R_2 = CH_3, R_3 = H, R_4 = H, R_5 = CH_2OH, R_6 = H, R_7 = H, n = 1, m =1, Y = 3-pyridinyl).

EXAMPLE 25

(Z)-5-Fluoro-2-Methyl-(2-Pyridinylidene)-3-(N-(S-α-Hydroxymethyl)Benzyl)-Indenylacetamide

5-Fluoro-2-methyl-3-(N-(S-α-hydroxylmethyl)benzyl)-indenylacetamide from Example 23 part A is allowed to react with 2-pryidinecarboxaldehyde according to the procedure of Example 1, Part G in order to obtain the title compound. Recrystallization gives the title compound (R_1 = F, R_2 = CH_3, R_3 = H, R_4 = H, R_5 = CH_2OH, R_6 = H, R_7 = H, n = 1, m =1, Y = 2-pyridinyl).

EXAMPLE 26

(Z)-5-Fluoro-2-Methyl-(4-Quinolinylidene)-3-(N-(S-α-Hydroxymethyl)Benzyl)-Indenylacetamide

5-Fluoro-2-methyl-3-(N-(S-α-hydroxylmethyl)benzyl)-indenylacetamide from Example 23 part A is allowed to react with 4-quinolinecarboxaldehyde according to the procedure of Example 1, Part G in order to obtain the title compound. Recrystallization gives the title compound (R_1 = F, R_2 = CH_3, R_3 = H, R_4 = H, R_5 = CH_2OH, R_6 = H, R_7 = H, n = 1, m =1, Y = 4-quinolinyl).

EXAMPLE 27

(Z)-5-Fluoro-2-Methyl-(Pyrazidinylidene)-3-(N-(S-α-Hydroxymethyl)Benzyl)-Indenylacetamide

5-Fluoro-2-methyl-3-(N-(S-α-hydroxylmethyl)benzyl)-indenylacetamide from Example 23 part A is allowed to react with pryazidinecarboxaldehyde according to the procedure of Example 1, Part G in order to obtain the title compound. Recrystallization gives the title compound (R_1 = F, R_2 = CH_3, R_3 = H, R_4 = H, R_5 = CH_2OH, R_6 = H, R_7 = H, n = 1, m =1, Y = pyrazidinyl).

EXAMPLE 28

(Z)-5-Fluoro-2-Methyl-(3-Pyridazinylidene)-3-(N-(S-α-Hydroxymethyl)Benzyl)-Indenylacetamide

5-Fluoro-2-methyl-3-(N-(S-α-hydroxylmethyl)benzyl)-indenylacetamide from Example 23 part A is allowed to react with pryidazine-3-aldehyde according to the procedure of Example 1, Part G in order to obtain the title compound. Recrystallization gives the title compound (R_1 = F, R_2 = CH_3, R_3 = H, R_4 = H, R_5 = CH_2OH, R_6 = H, R_7 = H, n = 1, m =1, Y = 3-pyridazinyl).

EXAMPLE 29

(Z)-5-Fluoro-2-Methyl-(4-Pyrimidinylidene)-3-(N-(S-α-Hydroxymethyl)Benzyl)-Indenylacetamide

5-Fluoro-2-methyl-3-(N-(S-α-hydroxylmethyl)benzyl)-indenylacetamide from Example 23 part A is allowed to react with pryimidine-4-aldehyde according to the procedure of Example 1, Part G in order to obtain the title compound. Recrystallization gives the title compound (R_1 = F, R_2 = CH_3, R_3 = H, R_4 = H, R_5 = CH_2OH, R_6 = H, R_7 = H, n = 1, m =1, Y = 4-pyrimidinyl).

EXAMPLE 30

(Z)-5-Fluoro-2-Methyl-(4-Pyridazinylidene)-3-(N-(S-α-Hydroxymethyl)Benzyl)-Indenylacetamide

5-Fluoro-2-methyl-3-(N-(S-α-hydroxylmethyl)benzyl)-indenylacetamide from Example 23 part A is allowed to react with pryidazine-4-aldehyde according to the procedure of Example 1, Part G in order to obtain the title compound. Recrystallization gives the title compound (R_1 = F, R_2 = CH_3, R_3 = H, R_4 = H, R_5 = CH_2OH, R_6 = H, R_7 = H, n = 1, m =1, Y = 4-pyridazinyl).

EXAMPLE 31

rac-(Z)-5-Fluoro-2-Methyl-(4-Pyridinylidene)-3-(N-Benzyl)Indenyl-α-Hydroxyacetamide

(A) 5-fluoro-2-methyl-3-(N-benzyl-N-hydroxy)-indenylacetamide

To a mixture of N-benzylhydroxylamine hydrochoride (12 mmol) and Et_3N (22 mmol) in CH_2Cl_2 (100 ml) at 0° C is added a cold solution of 5-fluoro-2-methylindenyl-3-acetyl chloride (Example 1, Step E) (10 mmol) in CH_2Cl_2 (75 ml) over a period of 45-60 minutes. The mixture is warmed to room temperature and is stirred for 1 hour. The mixture is diluted with water (100 ml), and the organic layer is washed with HCl (2 x 25 ml) and brine (2 x 100 ml), dried ($MgSO_4$) and evaporated. The crude product is purified with flash chromatography to give the title compound.

(B) 5-Fluoro-2-methyl-3-(N-benzyl-N-mesyloxy)-indenylacetamide

To a solution of 5-fluoro-2-methyl-3-(N-benzyl-N-hydroxy)-indenylacetamide (5 mmol) in CH_2Cl_2 (25 ml) at 0° C is added triethylamine (5 mmol). The mixture is stirred for 10 minutes, and methanesulfonyl chloride (5.5 mmol) is added dropwise. The solution is stirred at 0° C for 2 hours, allowed to warm to room temperature, and stirred for another 2 hours. The organic layer is washed with water (2 x 20 ml), in HCl (15 ml), and brine (20 ml) and dried over $MgSO_4$. After rotary evaporation, the product is purified with flash chromatography to give the title compound.

(C) rac-5-Fluoro-2-methyl-3-(N-benzyl)-α-hydroxyindenylacetamide

To a solution of 5-fluoro-2-methyl-3-(N-benzyl-N-mesyloxy)-indenylacetamide (2 mmol) in CH_3CN/H_2O (12 ml. each) is added triethylamine (2.1 mmol) in CH_3CN (24 ml) over a period of 6 hours. The mixture is stirred overnight. The solvent is removed, and the residue diluted with ethyl acetate (60 ml), washed with water (4 x 20 ml), in HCl (15 ml), and brine (20 ml) and dried over $MgSO_4$.

After rotary evaporation, the product is purified by recrystallization to give the title compound.

(D) rac-(Z)-5-Fluoro-2-methyl-(4-pyridinylidene)-3-(N-benzyl)-indenyl-α-hydroxyacetamide is obtained from rac-5-fluoro-2-methyl-3-(N-benzyl)-α-hydroxyindenylacetamide using the procedure of Example 1, Part G (R_1 = F, R_2 = CH_3, R_3 = OH, R_4 = H, R_5 = H, R_6 = H, R_7 = H, n =1, m = 1, Y = 4-pyridinyl).

EXAMPLE 32

2-[(Z)-5-Fluoro-2-Methyl-(4-Pyridinylidene)-3-(N-Benzyl)-Indenyl]-Oxyacetamide

For Pfitzner-Moffatt oxidation, a solution of rac-(Z)-5-fluoro-2-methyl-(4-pyridinylidene)-3-(N-benzyl)-indenyl-α-hydroxyacetamide (1 mmol) in DMSO (5 ml) is treated with dicyclohexylcarbodiimide (3 mmol). The mixture is stirred overnight, and the solvent is evaporated. The crude product is purified by flash chromatography to give the title compound (R_1 = F, R_2 = CH_3, R_3 and R_4 together form C = O, R_5 = H, R_6 = H, R_7 = H, n =1, m = 1, and Y=4-pyridinyl).

EXAMPLE 33

rac-(Z)-5-Fluoro-2-Methyl-(4-Pyridinylidene)-3-(N-Benzyl)-Indenyl-α-(2-Propylamino)-Acetamide

(A) 5-Fluoro-2-methyl-3-(N-2-propyl-N-hydroxy)-indenylacetamide is obtained from 5-fluoro-2-methylindenyl-3-acetyl chloride (Example 1, Step E) using the procedure of Example 31, Part A and replacing N-benzylhydroxylamine hydrochloride with N-2-propyl hydroxylamine hydrochloride.

(B) 5-Fluoro-2-methyl-3-(N-2-propyl-N-mesyloxy)-indenylacetamide is obtained according to the procedure of Example 31, Part B.

(C) rac-5-Fluoro-2-methyl-3-(N-benzyl)-α-(2-propylamino)-acetamide. To 5-fluoro-2-methyl-3-(N-2-propyl-N-mesyloxy)-indenylacetamide (2 mmol) in CH_2Cl_2 (25 ml) at 0° C is added benzylamine (4.4 mmol) in CH_2Cl_2 (15 ml) over a period of 30 minutes. The resulting solution is stirred at 0° C for 1 hour, and at room temperature overnight. The solvent is removed, and the residue is treated with 1 N NaOH, and is extracted with CH_2Cl_2 (100 ml). The extract is washed with water (2 x 10 ml), and is dried over $MgSO_4$. After rotary evaporation, the product is purified by flash chromatography.

(D) rac-(Z)-5-Fluoro-2-methyl-(4-pyridinylidene)-3-(N-benzyl)-indenyl-α-(2-propylamino)-acetamide is obtained from rac-5-fluoro-2-methyl-3-(N-benzyl)-α-(2-

propylamino)-acetamide using the procedure of Example 1, Part G (R_1 = F, R_2 = CH_3, R_3 = isopropylamino, R_4 = H, R_5 = H, R_6 = H, R_7 = H, n = 1, m = 1, Y = 4-pyridinyl).

EXAMPLE 34

(Z)-6-Methoxy-2-Methyl-(4-Pyridinylidene)-3-(N-Benzyl)-Indenylacetamide

(A) Ethyl-2-Hydroxy-2-(p-Methoxyphenyl)-1-Methylpropionate

In a 500 ml. 3-necked flask is placed 36.2 g. (0.55 mole) of zinc dust, a 250 ml. addition funnel is charged with a solution of 80 ml. anhydrous benzene, 20 ml. of anhydrous ether, 80 g. (0.58 mole) of p-anisaldehyde and 98 g. (0.55 mole) of ethyl-2-bromoproplonate. About 10 ml. of the solution is added to the zinc dust with vigorous stirring, and the mixture is warmed gently until an exothermic reaction commences. The remainder is added dropwise at such a rate that the reaction mixture continues to reflux smoothly (ca. 30-35 min.). After addition is completed the mixture is placed in a water bath and refluxed for 30 minutes. After cooling to 0°, 250 ml. of 10% sulfuric acid is added with vigorous stirring. The benzene layer is extracted twice with 50 ml. portions of 5% sulfuric acid and washed twice with 50 ml. portions of water. The combined aqueous acidic layers are extracted with 2 x 50 ml. ether. The combined etheral and benzene extracts are dried over sodium sulfate. Evaporation of solvent and fractionation of the residue through a 6" Vigreux column affords 89g. (60%) of the product, ethyl-2-hydroxy-2-(p-methoxyphenyl)-1-methylpropionate, B.P. 165-160° (1.5 mm.).

(B) 6-Methoxy-2-methylindanone

By the method described in Vander Zanden, Rec. Trav. Chim., 68, 413 (1949), the compound from part A is converted to 6-methoxy-2-methylindanone.

Alternatively, the same compound can be obtained by adding α-methyl-β-(p-methoxylphenyl)propionic acid (15 g.) to 170 g. of polyphosphoric acid at 50° and heating the mixture at 83-90° for two hours. The syrup is poured into iced water. The mixture is stirred for one-half hour, and is extracted with ether (3X). The etheral solution is washed with water (2X) and 5% $NaHCO_3$ (5X) until all acidic material has been removed, and is dried over sodium sulfate. Evaporation of the solution gives 9.1 g. of the indanone as a pale yellow oil.

(C) (Z)-6-Methoxy-2-methyl-(4-pyridinylidene)-3-(N-benzyl)-indenylacetamide

In accordance with the procedures described in Example 1, parts D-G, this compound is obtained substituting 6-methoxy-2-methylindanone for 6-fluoro-2-methylindanone in part D of Example 1.

EXAMPLE 35

(Z)-5-Methoxy-2-Methyl-(4-Pyridinylidene)-3-(N-Benzyl)-Indenylacetamide

(A) Ethyl 5-Methoxy-2-Methyl-3-Indenyl Acetate

A solution of 13.4 g of 6-methoxy-2-methylindanone and 21 g. of ethyl bromoacetate in 45 ml. benzene is added over a period of five minutes to 21 g. of zinc amalgam (prepared according to Org. Syn. Coll. Vol. 3) in 110 ml. benzene and 40 ml. dry ether. A few cyrstals of iodine are added to start the reaction, and the reaction mixture is maintained at reflux temperature (ca. 65°) with external heating. At three-hour intervals, two batches of 10 g. zinc amalgam and 10 g. bromoester are added and the mixture is then refluxed for 8 hours. After addition of 30 ml. of ethanol and 150 ml. of acetic acid, the mixture is poured into 700 ml. of 50% aqueous acetic acid. The organic layer is separated, and the aqueous layer is extracted twice with ether. The combined organic layers are washed thoroughly with water, ammonium hydroxide and water. Drying over sodium sulfate, evaporation of solvent in vacuo followed by pumping at 80° (bath temperature)(1-2 mm.) gives crude ethyl-(1-hydroxy-2-methyl-6-methoxy-indenyl) acetate (ca. 18 g.).

A mixture of the above crude hydroxyester, 20 g. of p-toluenesulfonic acid monohydrate and 20 g. of anhydrous calcium chloride in 250 ml. toluene is refluxed overnight. The solution is filtered, and the solid residue is washed with toluene. The combined toluene solution is washed with water, sodium bicarbonate, water and then dried over sodium sulfate. After evaporation, the crude ethyl 5-methoxy-2-methyl-3-indenyl acetate is chromatographed on acid-washed alumina, and the product is eluted with petroleum ether-ether (v./v. 50-100%) as a yellow oil (11.8 g., 70%).

(B) (Z)-5-Methoxy-2-methyl-(4-pyridinylidene)-3-(N-benzyl)-indenylacetamide

In accordance with the procedures described in Example 1, parts E-G, this compound is obtained substituting ethyl-5-methoxy-2-methyl-3-indenyl acetate for 5-fluoro-2-methindenyl-3-acetic acid in Example 1, part E.

EXAMPLE 36

(Z)- α-5-Methoxy-2-Methyl-(4-Pyridinylidene)-3-(N-Benzyl)-Indenylpropionamide

(A) α-(5-Methoxy-2-methyl-3-indenyl)propionic acid

The procedure of Example 35, part (A) is followed using ethyl α-bromopropionate in equivalent quantities in place of ethyl bromoacetate used therein. There is obtained ethyl α-(1-hydroxy-6-methoxy-2-methyl-1-indanyl)propionate, which is dehydrated to ethyl α-(5-methoxy-2-methyl-3-indenyl)propionate in the same manner.

The above ester is saponified to give α-(5-methoxy-2-methyl-3-indenyl)propionic acid.

(B) (Z)-α-5-Methoxy-2-methyl-(4-pyridinyl)-3-(N-benzyl)-α-methyl indenylpropionamide

In accordance with the procedures described in Example 1, parts E-G, this compound is obtained substituting α-5-methoxy-2-methyl-3-indenyl)propionic acid for 5-fluoro-2-methylindenyl-3-acetic acid in Example 1, part E.

EXAMPLE 37

(Z) α-Fluoro-5-Methoxy-2-Methyl-(4-Pyridinylidene)-3-(N-Benzyl)Indenylacetamide

(A) Methyl-5-Methoxy-2-Methyl-3-Indenyl-α-Fluoro Acetate

A mixture of potassium fluoride (0.1 mole) and methyl-5-methoxy-2-methyl-3-indenyl-α-tosyloxy acetate (0.05 mole) in 200 ml. dimethylformamide is heated under nitrogen at the reflux temperature for 2-4 hours. The reaction mixture is cooled, poured into iced water and then extracted with ether. The ethereal solution is washed with water, sodium bicarbonate and dried over sodium sulfate. Evaporation of the solvent and chromatography of the residue on an acid-washed alumina column (300 g.) using ether-petroleum ether (v./v. 20-50%) as eluent give the product, methyl-5-methoxy-2-methyl-3-indenyl-α-fluoroacetate.

(B) (Z) α-Fluoro-5-methoxy-2-methyl-(4-pyridinylidene)-3-(N-benzyl)indenylacetamide

In accordance with the procedures described in Example 1, parts E-G, this compound is obtained substituting methyl-5-methoxy-2-methyl-3-indenyl-α-fluoroacetate for 5-fluoro-2-methylindenyl-3-acetic acid in Example 1, part E.

For the introduction of the =CH-Y part in Scheme III, any of the appropriate heterocyclic aldehydes may be used either directly in the base-catalyzed condensation

or in a Wittig reaction in an alternative route. The aldehydes that may be used are listed in Table 1 below:

TABLE 1

pyrrol-2-aldehyde*

pyrimidine-2-aldehyde

6-methylpyridine-2-aldehyde*

1-methylbenzimidazole-2-aldehyde

isoquinoline-4-aldehyde

4-pyridinecarboxaldehyde*

3-pyridinecarboxaldehyde*

2-pyridinecarboxaldehyde*

4,6-dimethyl-2-pyridinecarboxaldehyde*

4-methyl-pyridinecarboxaldehyde*

4-quinolinecarboxaldehyde*

3-quinolinecarboxaldehyde*

2-quinolinecarboxaldehyde*

2-chloro-3-quinolinecarboxaldehyde*

pyrazinealdehyde

(Prepared as described by Rutner et al., JOC 1963, 28, 1898-99)

pyridazine-3-aldehyde

(Prepared as described by Heinisch et al., Monatshefte Fuer Chemie 108, 213-224,1977)

pyrimidine-4-aldehyde

(Prepared as described by Bredereck et al., Chem. Ber. 1964, 97, 3407-17)

2-methyl-pyrimidine-4-aldehyde

(Prepared as described by Bredereck et al., Chem. Ber. 1964, 97, 3407-17)

pyridazine-4-aldehyde

(Prepared as described by Heinisch et al., Monatshefte Fuer Chemie 104, 1372-1382 (1973))

1-methylindole-3-carboxaldehyde*

1-acetyl-3-indolecarboxaldehyde*

* Available from Aldrich

The aldehydes above can be used in the reaction schemes above in combination with various appropriate amines to produce compounds with the scope of this invention. Examples of appropriate amines are those listed in Table 2 below:

TABLE 2

benzylamine

2,4-dimethoxybenzylamine

2-methoxybenzylamine

2-fluorobenzylamine

4-dimethylaminobenzylamine

4-sulfonaminobenzylamine

1-phenylethylamine (R-enantiomer)

2-amino-2-phenylethanol (S-enantiomer)

2-phenylglycinonitrile (S-enantiomer)

EXAMPLE 38

(Z)-5-Fluoro-2-Methyl-(4-Pyridylidene)-3-(N-Benzyl) Indenylacetamide Hydrochloride

(Z)-5-Fluoro-2-methyl-(4-pyridylidene)-3-(N-benzyl)indenylacetamide (1396g ; MW 384.45; 3.63 mol) from Example 1 is dissolved at 45^0C in ethanol (28 L). Aqueous HCl (12 M; 363 mL) is added stepwise. The reaction mixture is heated under reflux for 1 hour, is allowed to cool to room temperature, then stored at -10^0C for 3 hours. The resulting solid is filtered off, is washed with ether (2 x 1.5 L) and is air-dried overnight. Drying under vacuum at 70^0C for 3 days gives (Z)-5-fluoro-2-methyl-(4-pyridylidene)-3-(N-benzyl)indenylacetamide hydrochloride with a melting point of 207-209^0C (R_1 = F, R_2 = CH_3, R_3 = H, R_4 = H, R_5 = H, R_6 = H, R_7 = H, n = 1, m = 1, Y = 4-pyridinyl • hydrochloride). Yield: 1481 g (97 %; 3.51 mol); MW: 420.91 g/mol.

^{1}H-NMR (DMSO-d_6): 2.18 (s,3,=C-CH_3); 3.54 (s,2,=CH_2CO); 4.28 (d,2,NCH_2); 6.71 (m,1,ar.); 7.17 (m,8,ar.); 8.11 (d,2,ar., AB system); 8.85 (m,1,NH); 8.95 (d,2,ar.,AB system); IR (KBr): 3432 NH; 1635 C=O; 1598 C=C.

EXAMPLE 39

(Z)-5-fluoro-2-methyl-(4-pyridylidene)-3-(N-benzyl)-indenylacetamide p-methylbenzenesulfonate

(Z)-5-fluoro-2-methyl-(4-pyridylene)-3-(N-benzyl)indenylacetamide (MW=384.46 g/mol; 5.21 mmol; 2 g) from Example 1 is dissolved in ethanol (50 ml).

Solid p-toluenesulfonic acid monohydrate (MW=190.22 g/mol; 5.21 mmol; 991 mg) is added to the stirred solution. The reaction mixture is stirred for 12 hours at room temperature. The ethanol is evaporated in aspirator vacuum. The residue is dried in high vacuum to yield (Z)-5-fluoro-2-methyl-(4-pyridylidene)-3-(N-benzyl)-indenylacetamide p-methylbenzenesulfonate as an orange-red powder.

As to identifying structurally additional PDE2 and PDE5 inhibiting compounds besides those of Formula I that can be effective therapeutically for lupus erythematosus, one skilled in the art has a number of useful model compounds disclosed herein (as well as their analogs) that can be used as the bases for computer modeling of additional compounds having the same conformations but different chemically. For example, software such as that sold by Molecular Simulations Inc. release of WebLab® ViewerPro™ includes molecular visualization and chemical communication capabilities. Such software includes functionality, including 3D visualization of known active compounds to validate sketched or imported chemical structures for accuracy. In addition, the software allows structures to be superimposed based on user-defined features, and the user can measure distances, angles, or dihedrals.

In this situation, since the structures of active compounds are disclosed above, one can apply cluster analysis and 2D and 3D similarity search techniques with such software to identify potential new additional compounds that can then be screened and selected according to the selection criteria of this invention. These software methods rely upon the principle that compounds, which look alike or have similar properties, are more likely to have similar activity, which can be confirmed using the PDE selection criterion of this invention.

Likewise, when such additional compounds are computer-modeled, many such compounds and variants thereof can be synthesized using known combinatorial chemistry techniques that are commonly used by those of ordinary skill in the pharmaceutical industry. Examples of a few for-hire combinatorial chemistry services include those offered by New Chemical Entities, Inc. of Bothell Washington, Protogene Laboratories, inc., of Palo Alto, California, Axys, Inc. of South San Francisco, California, Nanosyn, Inc. of Tucson, Arizona, Trega, Inc. of San Diego, California, and RBI, Inc. of Natick, Mass. There are a number of other for-hire companies. A number of large pharmaceutical companies have similar, if not superior, in-house capabilities. In short, one skilled in the art can readily produce many compounds for screening from which to

select promising compounds for treatment of neoplasia having the attributes of compounds disclosed herein.

To further assist in identifying compounds that can be screened and then selected using the criterion of this invention, knowing the binding of selected compounds to PDE5 and PDE2 protein is of interest. By the procedures discussed below, it is believed that that preferable, desirable compounds meeting the selection criteria of this invention bind to the cGMP catalytic regions of PDE2 and PDE5.

To establish this, a PDE5 sequence that does not include the catalytic domain can be used. One way to produce such a sequence is to express that sequence as a fusion protein, preferably with glutiathione S-transferase ("GST"), for reasons that will become apparent.

RT-PCR method is used to obtain the cGB domain of PDE5 with forward and reverse primers designed from bovine PDE5A cDNA sequence (*McAllister-Lucas L. M. et al, J. Biol. Chem. 268, 22863-22873, 1993)* and the selection among PDE 1-10 families. 5'-3', Inc. kits for total RNA followed by oligo (dT) column purification of mRNA are used with HT-29 cells. Forward primer (GAA-TTC-TGT-TAG-AAA-AGC-CAC-CAG-AGA-AAT-G, 203-227) and reverse primer (CTC-GAG-CTC-TCT-TGT-TTC-TTC-CTC-TGC-TG, 1664-1686) are used to synthesize the 1484 bp fragment coding for the phosphorylation site and both low and high affinity cGMP binding sites of human PDE5A (203-1686 bp, cGB-PDE5). The synthesized cGB-PDE5 nucleotide fragment codes for 494 amino acids with 97% similarity to bovine PDE5A. It is then cloned into pGEX-5X-3 glutathione-S-transferase (GST) fusion vector (Pharmacia Biotech)with tac promoter, and EcoRI and XhoI cut sites. The fusion vector is then transfected into E. Coli BL21 (DE3) bacteria (Invitrogen). The transfected BL21 bacteria is grown to log phase, and then IPTG is added as an inducer. The induction is carried at 20°C for 24 hrs. The bacteria are harvested and lysed. The soluble cell lysate is incubated with GSH conjugated Sepharose 4B (GSH-Sepharose 4B). The GST-cGB-PDE5 fusion protein can bind to the GSH-Sepharose beads, and the other proteins are washed off from beads with excessive cold PBS.

The expressed GST-cGB-PDE5 fusion protein is displayed on 7.5% SDS-PAGE gel as an 85 Kd protein. It is characterized by its cGMP binding and phosphorylation by protein kinases G and A. It displays two cGMP binding sites, and the K_d is 1.6±0.2 μM, which is close to K_d=1.3 μM of the native bovine PDE5. The GST-cGB-PDE5 on GSH-

conjugated sepharose beads can be phosphorylated *in vitro* by cGMP-dependent protein kinase and cAMP-dependent protein kinase A. The K_m of GST-cGB-PDE5 phosphorylation by PKG is 2.7μM and Vmax is 2.8 μM, while the K_m of BPDEtide phosphorylation is 68μM. The phosphorylation by PKG shows molecular phosphate incorporated into GST-cGB-PDE5 protein on a one-to-one ratio.

A cGMP binding assay for compounds of interest (Francis S. H. et al, J. Biol. Chem. 255, 620-626, 1980) is done in a total volume of 100 μL containing 5 mM sodium phosphate buffer (pH=6.8), 1 mM EDTA, 0.25 mg/mL BSA, H^3-cGMP (2μM, NEN) and the GST-cGB-PDE5 fusion protein (30 μg /assay). Each compound to be tested is added at the same time as ^{3}H-cGMP substrate, and the mixture is incubated at 22°C for 1 hour. Then, the mixture is transferred to Brandel MB-24 cell harvester with GF/B as the filter membrane followed by 2 washes with 10 mL of cold 5 mM potassium buffer(pH 6.8). The membranes are then cut out and transferred to scintillation vials followed by the addition of 1 mL of H_2O and 6 mL of Ready Safe™ liquid scintillation cocktail to each vial. The vials are counted on a Beckman LS 6500 scintillation counter.

For calculation, blank samples are prepared by boiling the binding protein for 5 minutes, and the binding counts are < 1% when compare to unboiled protein. The quenching by filter membrane or other debris are also calibrated.

PDE5 inhibitors, sulindac sulfide , exisulind, E4021 and zaprinast, and cyclic nucleotide analogs, cAMP, cyclic IMP, 8-bromo-cGMP, cyclic UMP, cyclic CMP, 8-bromo-cAMP, 2'-O-butyl-cGMP and 2'-O-butyl-cAMP were selected to test whether they could competitively bind to the cGMP binding sites of the GST-cGB-PDE5 protein. cGMP specifically bound to GST-cGB-PDE5 protein. Cyclic AMP, cUMP, cCMP, 8-bromo-cAMP, 2'-O-butyl-cAMP and 2'-O-butyl-cGMP did not compete with cGMP in binding. Cyclic IMP and 8-bromo-cGMP at high concentration (100 μM) can partially compete with cGMP (2 μM) binding. None of the PDE5 inhibitors showed any competition with cGMP in binding of GST-cGB-PDE5. Therefore, they do not bind to the cGMP binding sites of PDE5.

However, Compound 38 does competitively (with cGMP) bind to PDE 5. Given that Compound 38 does not bind to the cGMP-binding site of PDE5, the fact that there is competitive binding between Compound 38 and cGMP at all means that desirable compounds such as Compound 38 bind to the cGMP catalyic site on PDE5, information that is readily obtainable by one skilled in the art (with conventional competitive binding

experiments) but which can assist one skilled in the art more readily to model other compounds. Thus, with the chemical structures of desirable compounds presented herein and the cGMP binding site information, one skilled in the art can model, identify and select (using the selection criteria of this invention) other chemical compounds for use as therapeutics.

Examples of compounds that inhibit PDE2 and PDE5 (with insubstantial COX inhibition) include exisulind and compounds disclosed in U.S. Patents Nos. 5,965,619 and 6,063,818 which are incorporated herein by reference.

BIOLOGICAL EFFECTS

(A) Cyclooxygenase (COX) Inhibition

COX catalyzes the formation of prostaglandins and thromboxane by the oxidative metabolism of arachidonic acid. The compound of Example 1 of this invention, as well as a positive control, (sulindac sulfide) were evaluated to determine whether they inhibited purified cyclooxygenase Type I (see Table 1 below).

The compounds of this invention were evaluated for inhibitory effects on purified COX. The COX was purified from ram seminal vesicles, as described by Boopathy, R. and Balasubramanian, J., 239:371-377, 1988. COX activity was assayed as described by Evans, A.T., et al., "Actions of Cannabis Constituents on Enzymes Of Arachidonate Metabolism Anti-Inflammatory Potential," Biochem. Pharmacol., 36:2035-2037, 1987. Briefly, purified COX was incubated with arachidonic acid (100 μM) for 2.0 min at 37°C in the presence or absence of test compounds. The assay was terminated by the addition of TCA, and COX activity was determined by absorbance at 530 nm.

TABLE 1

EXAMPLE	COX I % Inhibition(100μM)
Sulindac sulfide	86
1	<25

The advantage of very low COX inhibition is that compounds of this invention can be administered to patients without the side effects normally associated with COX inhibition.

(B) cGMP PDE Inhibition

Compounds of this invention are also PDE2 and PDE5 inhibitors as taught in part U.S. Patent Application Serial No. 09/046,739 filed March 24, 1998. Compounds can be tested for inhibitory effect on phosphodiesterase activity using either the enzyme isolated from any tumor cell line such as HT-29 or SW-480. Phosphodiesterase activity can be determined using methods known in the art, such as a method using radioactive ^3H cyclic GMP (cGMP)(cyclic 3',5'-guanosine monophosphate) as the substrate for PDE5 enzyme. (Thompson, W.J., Teraski, W.L., Epstein, P.M., Strada, S.J., *Advances in Cyclic Nucleotide Research*, 10:69-92, 1979, which is incorporated herein by reference). In brief, a solution of defined substrate ^3H-cGMP specific activity (0.2 μM; 100,000 cpm; containing 40 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$ and 1 mg/ml BSA) is mixed with the drug to be tested in a total volume of 400μl. The mixture is incubated at 30°C for 10 minutes with partially purified cGMP-specific PDE isolated from HT-29 cells. Reactions are terminated, for example, by boiling the reaction mixture for 75 seconds. After cooling on ice, 100 μl of 0.5 mg/ml snake venom (*O. Hannah* venom available from Sigma) is added and incubated for 10 min at 30°C. This reaction is then terminated by the addition of an alcohol, e.g. 1 ml of 100% methanol. Assay samples are applied to a anion chromatography column (1 ml Dowex, from Aldrich) and washed with 1 ml of 100% methanol. The amount of radioactivity in the breakthrough and the wash from the columns in then measured with a scintillation counter. The degree of PDE5 inhibition is determined by calculating the amount of radioactivity in drug-treated reactions and comparing against a control sample (a reaction mixture lacking the tested compound).

Using such protocols, the compound of Example 1 had an IC_{50} value for PDE5 inhibition of 0.68 μM. Using similar protocols, the compound of Example 38 ("Compound 38") had an IC_{50} value for PDE2 of 14 μM, an IC_{50} value for PDE5 of 4 μM, an IC_{50} value for PDE1 of 3 μM, and an IC_{50} value for PDE4 of 6 μM.

(C) Safety Assessment in Mammals

As one skilled in the art will recognize from the data presented below, Compound 38 can safely be given to animals at doses far beyond the tolerable (and in many cases

toxic) doses of non-insulin lupus erythematosus therapies. For example, in an acute toxicity study in rats, single oral doses of Compound 38 administered (in a 0.5% carboxy-methylcellulose vehicle) at doses up to and including 2000 mg/kg resulted in no observable signs of toxicity. At 2000 mg/kg, body weight gains were slightly reduced. A single dose of 1000 mg/kg administered intraperitoneally resulted in reduced body weight gain, with mesenteric adhesions seen in some animals from this group at necropsy.

In dogs, the administration of Compound 38 in capsules at 1000 mg/kg resulted in no signs of toxicity to the single group of two male and two female dogs. Due to the nature of Compound 38 capsules, this dose necessitated the use of at least 13 capsules to each animal, which was judged to be the maximum number without subjecting the animals to stress. Therefore, these dogs were subsequently administered seven consecutive doses of 1000 mg/kg/day. At no time in either dosing phase were any obvious signs of drug-related effects observed.

Thus, on a single-dose basis, Compound 38 is not acutely toxic. Based on the findings of these studies, the oral LD_{50} of Compound 38 was considered to be greater than 1000 mg/kg in dogs and 2000 mg/kg in rats, and the intraperitoneal LD_{50} was considered to be greater than 1000 mg/kg in rats.

A seven-day dose-range finding study in rats, where Compound 38 was evaluated by administering it at doses of 0, 50, 500 or 2000 mg/kg/day resulting in no observable signs of toxicity at 50 mg/kg/day. At 500 mg/kg/day, treatment-related effects were limited to an increase in absolute and relative liver weights in female rats. At 2000 mg/kg/day, effects included labored breathing and/or abnormal respiratory sounds, decreased weights gains and food consumption in male rats, and increased liver weights in female rats. No hematological or blood chemistry changes nor any microscopic pathology changes, were seen at any dose level.

A 28-day study in rats was also carried out at 0, 50, 500 and 2000 mg/kg/day. There were no abnormal clinical observations attributed to Compound 38, and body weight changes, ophthalmoscopic examinations, hematological and blood chemistry values and urinalysis examinations were unremarkable. No macroscopic tissue changes were seen at necropsy. Organ weight data revealed statistically significant increase in liver weights at 2000 mg/kg/day, and statistically significant increases in thyroid weights for the 2000 mg/kg/day group. The slight liver and thyriod increases at the lower doses were not statistically significant. Histopathological evaluation of tissues indicated the presence of traces of follicular cell hypertrophy, increased numbers of mitotic figures

(suggestive of possible cell proliferation) in the thyroid gland and mild centrilobular hypertrophy in the liver. These changes were generally limited to a small number of animals at the 2000 mg/kg/day dose, although one female at 500 mg/kg/day had increased mitotic figures in the thyroid gland. The findings in the liver may be indicative of a very mild stimulation of liver microsomal enzymes, resulting in increased metabolism of thyroid hormones, which in turn resulted in thyroid stimulation.

A long-term safety assessment study was conducted in rats to investigate Compound 38 at 50, 200 and 500 mg/kg/day following repeated oral dosing for 91 consecutive days. Orally administered Compound 38 did not produce any major toxicological effects in rats. The only finding was a dose-related trend to increased liver and thyroid/parathyroid weights noted in males and females at 200 and 500 mg/kg/day. Microscopically, slight hepatocellular hypertrophy at 200 and 500 mg/kg/day groups, follicular cell hypertrophy at 500 mg/kg/day and increase in accumulation of hyalin droplets in the kidneys at 200 and 500 mg/kg/day group. However, no changes in clinical biochemistry and hematology were evident. These changes were not associated with any gross clinical abnormality.

Dogs were also dosed orally with Compound 38 at 50, 150 and 300 mg/kg/day for 91 consecutive days. There were no toxicological effects in the dog following 91 days of dosing. Orange discoloration of the feces (same color as Compound 38) was seen in the 150 and 300 mg/kg/day groups. This finding suggested that most of Compound 38 was being eliminated via the feces. Slightly lowered body weights were noted in the highest dose group. This dose was also associated with increased liver weights. However, there were no microscopic alterations to support the increase in liver weight. Therefore, we concluded that Compound 38 is well tolerated in the dog.

Finally as to safety, in a single, escalating dose human clinical trial, patients, human safety study in which the drug was taken orally, Compound 38 produced no significant side effects at any dose (i.e., 50 mg BID, 100 mg BID, 200 mg BID and 400 mg BID). -- doses above the level believed to be therapeutic for human patients.

One skilled in the art should recognize that any of the side effects observed in these safety studies occurred at very high doses, in excess of recommended human doses and are extremely minimal compared to what one would expect at similar doses of other proposed therapies.

(D) Efficacy for lupus erythematosus

i. Macrophage Involvement

As mentioned above, macrophages have been implicated in the progression of lupus according to Lee MS et al., *Australas J. Dermatol.* 1996 Nov.; 37(4): 188-92. Specifically, macrophages have been found to invade skin (Tsukahara T et al., *Hiroshima J. Med. Sci.*, 1995 Mar; 44(1): 13-6).

As demonstrated below, we found that macrophages contain PDE2 and PDE5, and the inhibition of PDE2 particularly with PDE5 inhibition leads to apoptosis of macrophage cells. We believe the administration of a PDE2 inhibitor can treat the progression of lupus erythematosus, particularly when PDE5 is also inhibited.

ii. PDE2 and PDE5 mRNA Levels in Treated and Untreated U937 Cells by RT-PCR

The U937 monocyte cell line was derived from a histocytic lymphoma and can be driven to differentiate into an 'activated macrophage like' state by treatment with 5nM phorbal ester (TPA). Treated U937 cells become adherent, increase their cytoplasmic volume and express macrophage-specific cell surface markers. The presence and level of PDE2 and 5 mRNA in both differentiated and non-differentiated U937 cells was confirmed by performing RT-PCR experiments on total RNA.

U937 cells (from ATCC Rockville, MD) were grown in RPMI media supplemented with 5% FCS, glutamine, antibiotic/antimycotic and sodium pyruvate. Total RNA was isolated from two U937 cultures, one treated with 5nM TPA for 48 hours and one grown in normal media as listed above, using the Rouche High Pure RNA Isolation Kit (cat# 1 828 665) as per manufacturers protocol. cDNA was then synthesized from the total RNA using GibcoBRL SuperscriptII (Cat # 18064-022) reverse transcriptase as per manufacturers protocol. The resulting cDNA was used as a template for RT-PCR reactions using primer sets specific for PDE2 (forward: CCCAAAGTGGAGACTGTCTACACCTAC, reverse: CCGGTTGTCTTCCAGCGTGTC) or PDE5 (forward: GGGACTTTACCTTCTCATAC, reverse: GTGACATCCAAATGACTAGA). mRNA for PDE2 and 5 were both present in the untreated U937 cells. Upon treatment with TPA, the relative amounts of PDE2 mRNA increased 5 fold. Therefore, U937 cells treated with TPA and driven to differentiate into an activated macrophage like state have elevated levels of PDE2 mRNA (see Figure 1).

iii. Confirmation of PDE2 and PDE5 Protein Within U937 Cells by Indirect Immunofluorescence

The presence of PDE2 and PDE5 protein within U937 cells was confirmed by indirect immunofluorescence (IIF). U937 cells were cultured as above. Two U937 cultures, one grown in the presence of 5nM TPA for 48 hours and one grown in normal media were processed. All cultures were collected by centrifugation (Shandon Cytospin, 2 minutes @ 600 rpm) onto poly-L lysine-coated slides and immediately fixed in fresh 3% paraformaldehyde buffered in PBS for 10 minutes. Adherent cultures were grown on coverslips and fixed as above. Cells were permeablized in 0.2% triton-100 for 2 minutes. Slides were blocked with blocking buffer (5% goat serum, 5% glycerol, 1% gelatin from cold water fish skin and 0.04% NaN_3 in PBS) for 1 hour at room temperature.

Slides were then incubated for 1 hour at 37°C in a humid chamber with antibodies specific for PDE2 (generated in a sheep against the peptide TLAFQKEQKLKCECQA) or PDE5 (generated in sheep against the peptide CAQLYETSLLENKRNQV). The PDE5 antibody was used at a dilution of 1:200 and the PDE2 antibody was used at a dilution of 1:100. All dilutions were performed in blocking buffer. Slides were then washed 2x for 10 minutes each in PBS and then incubated with a Cy3 conjugated secondary antibody (Jackson ImmunoResearch laboratories, Inc. Cat. # 713-166-147) diluted 1:1000 in blocking buffer, for 1 hour at 37°C in a humid chamber. Slides were then washed 2x for 10 minutes each in PBS and counterstained with DAPI (5 ng/ml) and mounted in VectaShield. Digital images were then obtained using a SPOT-2 camera and an Olympus IX-70 fluorescent microscope. Both PDE2 and PDE5 are present in the cytoplasm of U937 cells. There is an increase in the level of both PDE2 and PDE5 in TPA-treated U937 cells. These increased protein levels are seen in discrete perinuclear foci (see Figures 2 through 5).

iv. Cyclic GMP Hydrolysis Within U937 cells

cGMP-hydrolytic activity in TPA-treated and untreated U937 cells was determined by performing a permeablized cell assay and direct analysis of enzyme activity in protein lysates. Both procedures achieved similar results, namely, elevated activity in the treated cells compared to untreated cells.

The cGMP hydrolysis levels in permeablized U937 cells was performed by washing the cells for 5 minutes with DMEM followed by cold PBS. Cells were then

placed on ice in 700µl ice cold Tris-HCL buffer (20 mM; pH 7.4) containing $MgCl_2$ (5mM) 0.5% Triton X-100, and protein inhibitors (10mM bezamidine, 10µM TLDK, 2000U/ml aprotinin, 2µM leupeptin, 2µM pepstatin A). The reaction was initiated by the addition of 100 µl of 0.5 mg/ml snake venom and 0.25 µM cGMP or cAMP along with [^{3}H]cGMP or [^{3}H]cAMP, respectively. After incubating for 30 minutes at 30°C the reactions were terminated by the addition of 1.8 ml methanol. The extract was then applied to a 1 ml Dowex anion exchange column to remove unreacted substrate. The eluant was collected and counted in 6 ml scintillation fluid. As shown in Figure 6, U937 cell cGMP hydrolysis levels elevate when the cells are driven into an activated macrophage-like state upon treatment with TPA, as compared to unactivated, untreated cells.

cGMP hydrolysis levels in protein lysates extracted from TPA-treated and untreated U937 cells were also analyzed as follows. Cells were resuspended in 20mM TRIS-HCl, 5 mM MgCl2, 0.5% Triton X-100, 0.1 mM EDTA, 10 mM benzamidine, 10 µM TLCK, 20 nM aprotinin, 2 µM leupeptin, 2 µM pepstatin A, pH 8.0 were added. The cells were homogenized using a glass tissue grinder and teflon pestle. Samples were ultracentrifuged at 100,000 x g for 1 hr at 0°C. Supernatants were assayed at 0.25 µM cGMP using the method from Thompson, W. J. *et. al.* Adv. Cyclic Nucleotide Res., *10:* 69–92, 1979. Again, the level of cGMP hydrolytic activity increased upon TPA treatment/activation, compared with no treatment/unactivation (see Figure 7). Both of these experiments corroborate the results of our experiments above that show that both cGMP PDE2 and PDE5 protein levels increase in U937 cells treated with TPA.

v. Apoptosis Induction of U937 Cells by Compound 38

U937 cells were cultured, as described above, with and without treatment with 5nM TPA for 24 hours at which time the cultures were treated either with 1µM Compound 38 or vehicle (DMSO) alone for an additional 24 hours. Adherent cells were dislodged by treatment with trypsin EDTA for 5 minutes at 37°C. Cells were then processed for IIF as described above, except that an antibody specific for active caspase 3 was used (as per manufacturer's protocol) instead of antibodies to PDE2 or 5 (Promega Cat. #G7481). The anti-active caspase 3 antibody was diluted 1:200 in blocking buffer and processed according to the manufacturer's protocol. The resulting slides were observed under a fluorescent microscope and a digital images

were obtained. Figure 8 shows U937 cells treated with 1μM compound 38 undergoing apoptosis as reflected by the presence of active caspase 3 (red signal). Image of control (vehicle only) U937 cells reveals only low, background levels of apoptosis (Figure 9).

The level of apoptosis in U937 cells was quantified by scoring 500 consecutive cells for the presence of active caspase 3. These results are summarized in the following table.

Cell type	TPA treatment	Compound 38	Number of apoptotic cells	Percentage of apoptotic cells
U937			6/500	1.2%
U937		1uM, 24 hrs	375/500	75%
U937	5nM, 16hrs		59/500	11.8%
U937	5nM, 16hrs	1uM, 24hrs	392/500	78%

Therefore, compound 38 causes the induction of apoptosis in the differentiated and non-differentiated U937 cell line.

vi. Treatment of U937 Cells With Either Sildenafil (PDE5-Specific Inhibitor) or Rolipram (PDE4-Specific Inhibitor) Does Not Induce Apoptosis.

The activity of specific PDE inhibitors contrast with the activity of compound 38 in U937 cells. By "specific" in this context, we mean the other PDE inhibitors that inhibited one PDE primarily, but not several PDEs (e.g., inhibiting PDE2 and PDE5 at roughly the same concentration). An example is sildenafil, which primarily inhibits PDE5, and only at much higher concentrations may only marginally inhibit other PDEs. Another example is rolipram (PDE4-specific).

U937 cells were incubated in the presence of 0.3nM sildenafil or 0.5uM rolipram for 24 hours using the culture conditions described above. The cells were harvested and processed for IIF as described above using an antibody that specifically recognizes active caspase 3. Digital images are shown in Figures 10 and 11. No increase in the levels of apoptosis compared to normal background was observed.

Therefore, the inhibition of only PDE4 or PDE5 alone (i.e. without the inhibition of PDE2) is not sufficient to induce apoptosis in U937 cells.

vii. Compound 38 Decreases TNF Alpha Levels in U937 Media

One function of macrophages is to modulate the activity of other inflammatory cells through various cytokine molecules. We therefore tested the effect of compound 38 on the ability of U937 cells to produce and secrete tumor necrosis factor-α (TNF-α). This was done by performing an immunoassay on the cell culture media taken from differentiated U937 cells (TPA treated) grown in the presence or absence of compound 38.

TNF-α levels in the cell culture media were determined by using the TNF-α Immunoassay from R&D Systems (Cat. # DTA50) according to the manufacturer's protocol. As shown in Figure 12, Compound 38 treatment significantly reduced the level of TNF-α secreted by TPA-induced U937 cells.

viii. Human Discoid Lupus Erythematosus

Skin tissue was obtained from a patient with a known history of discoid lupus erythematosus. Tissue was formalin-fixed, processed and sections at a thickness of 5μm. A serial dilution study demonstrated the optimal signal-to-noise ratio was 1:100 and 1:200 (PDE2), 1:500 and 1:1000 (PDE5). Anti-PDE2 and anti-PDE5 was used as the primary antibodies, and the principal detection system consisted of a Vector anti-sheep secondary (BA-6000) and Vector ABC-AP Kit (AK-5000) with a Vector Red substrate kit (SK-5100), which was used to produce a fuchsia-colored red deposit. Tissues were also stained with a positive control antibody (CD31) to ensure the tissue antigens were preserved and accessible for immunohistochemical analysis. CD31 is present in monocytes, macrophages, granulocytes, B lymphocytes and platelets. The negative control consisted of performing the entire immunohistochemistry procedure on adjacent sections in the absence of primary antibody. Slides were imaged using a DVC Digital Photo Camera coupled to a Nikon microscope.

As shown in Figures 13-15, human skin discoid lupus erythematosus tissue samples exhibited positive staining for PDE2 and PDE5 immunoactivity that was mostly localized to macrophages, lymphocytes and neutrophils. Figures 13 and 14 are visual images of immunostaining to PDE2 protein and figure 15 is a visual image of immunostaining to PDE5 protein.

It will be understood that various changes and modifications can be made in the details of procedure, formulation and use without departing from the spirit of the invention, especially as defined in the following claims.